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# THE ROUTE OF INFECTION INFLUENCES THE CONTRIBUTION OF KEY IMMUNITY GENES TO ANTIBACTERIAL DEFENSE IN ANOPHELES GAMBIAE

by
AMIRA SAN IMAD DEKMAK

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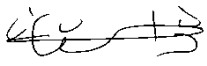
AMIRA SAN IMAD DEKMAK

Approved by:

Dr. Mike Osta, Associate Professor — Advisor
Department of Biology
American University of Beirut

Dr. Sawsan Kreydiyyeh, Professor — Member of Committee
Department of Biology
American University of Beirut

Dr. Laure El Chamy, Associate Professor — Member of Committee
Department of Life and Earth Sciences
Saint Joseph University



Dr. Dani Osman, Associate Professor — Member of Committee
Faculty of Sciences III
Lebanese University

Dr. Zakaria Kambris, Associate Professor — Member of Committee
Department of Biology
American University of Beirut

Date of defense: October 16, 2020

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# ACKNOWLEDGEMENTS

*"And once the storm is over, you won't remember how you made it through, how you managed to survive. You won't even be sure, whether the storm is really over. But one thing is certain. When you come out of the storm, you won't be the same person who walked in. That's what this storm's all about."* **Haruki Murakami**

It is not easy for me to realize that this journey has come to an end. I walked in with all my heart but am not leaving the same way.

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Daughter, you were the sweetest company during my last year. Believe in yourself if one day someone whispers to you "You can't withstand the storm". You can.

# ABSTRACT
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Title: The route of infection influences the contribution of key immunity genes to antibacterial defense in *Anopheles gambiae*

Pathogens gain access to their hosts through several routes, most of which require contact with barrier epithelia Indeed, studies in insects and mammals suggest that different routes of infection are likely to trigger different physiological responses in the host. However, the nature of these responses and how they impact host resistance and tolerance in different infection routes is not completely understood. Immunity studies in several model insects have focused largely on conducting microbial challenges using microinjections, whereby the microbe is directly injected into the hemolymph, a scenario that is less likely to occur in nature and one that bypasses the multilayered immune response elicited following microbial colonization and invasion of barrier epithelial. Hence, it remains unclear whether and to what extent the contribution of systemic immune defenses to host resistance to infection varies if bacteria invade the hemolymph after crossing the midgut epithelium subsequent to an oral infection. Here, we address this question using the pathogenic *Serratia marcescens* (*Sm*) DB11 strain to establish systemic infection of the hemolymph in the malaria vector *Anopheles gambiae*, either by septic *Sm* injections or by midgut crossing after feeding on *Sm*. Indeed, we were able to detect *Sm* in the mosquito hemolymph one day after oral infection, a clear indication that *Sm* is able to cross the midgut epithelial barrier and gain access to the body cavity. Using functional genetic studies by RNA interference (RNAi), we report that the two humoral immune factors, thioester-containing protein 1 (TEP1) and C-type lectin 4 (CTL4), which play key roles in defense against Gram-negative bacterial infections, are essential for defense against systemic *Sm* infections established through injection but they become dispensable when *Sm* infects the hemolymph following oral infection. Similar results were observed for the mosquito Relish 2 (Rel2)/Immune deficiency (Imd) pathway, indicating that this pathway may either be not activated in response to oral infection or that it is activated but rather non-essential for defense against *Sm* oral infection. Surprisingly, blocking phagocytosis by cytochalasin D treatment did not affect mosquito susceptibility to *Sm* infections established through either route. A plausible explanation could be that this cellular response is not essential when small numbers of bacteria are present in the hemolymph, as is the case with our infection protocol herein. Transcriptomic analysis of mosquito midguts and abdomens by RNA sequencing (RNA-seq) revealed that the transcriptional response in these tissues is more pronounced in response to feeding on *Sm*, despite the fact that injections resulted eventually in higher loads of *Sm* in the hemolymph. A small overlap was observed when comparing differentially expressed transcripts in midguts and abdomens of mosquitoes injected with *Sm* to those of mosquitoes fed on *Sm*,

indicating that different physiological responses are triggered in response to the different routes of *Sm* infection. Functional classification of all differentially expressed transcripts in abdomens and midguts from all treatments revealed that metabolic genes are the most represented class. Surprisingly, oral and septic infections with *Sm* seem to have little effect on the transcriptome of immunity genes as these were under-represented in both abdomens and midguts from all treatments. We also report that *Sm* oral infections are associated with significant downregulation of several immune genes belonging to different families, specifically the clip-domain serine protease family. On the other hand, only four immunity genes were upregulated after *Sm* oral infections; Galectin 5 and CecA were upregulated in abdomens, whereas, C-type lectin 6 (CTL6) and lysozyme C7 (LYSC7) were upregulated in the midgut. In sum, our findings reveal that the route of infection not only alters the contribution of key immunity genes to host anti-microbial defense, but is also associated with different transcriptional responses in midguts and abdomens, possibly reflecting different adaptive strategies of the host.

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# ABBREVIATIONS

| **Abbreviations** | **Definition** |
|---|---|
| Apo-II/I | Apolipoprotien II/I |
| 5C | Cytoplasmic actin |
| *Ae. aegypti* | *Aedes aegypti* |
| *A. albimanus* | *Anopheles albimanus* |
| *A. albopictus* | *Aedes albopictus* |
| *A. stephensi* | *Anopheles stephensi* |
| *A. gambiae* | *Anopheles gambiae* |
| AATI | Advanced Analytical Technologies Inc. |
| aDNA | Ancient Deoxyribonucleic acid |
| AGBP | Bacteria recognition family |
| AMPs | Antimicrobial peptides |
| AP-1 | Activator protein 1 |
| APL1C | *Anopheles Plasmodium*-responsive Leucine-rich repeat protein 1 |
| AZ | Organelle-free actin zone |
| b-GBPs | Beta-glucan-binding proteins |
| *B. bassiana* | Beauveria bassiana |
| BALB/c | Bagg Albino |
| BCL6 | Proto-oncogene B-cell lymphoma 6 |

| | |
|---|---|
| BH4 | Tetrahydrobiopterin |
| BINT2 | ß-integrin |
| BRWD3 | Bromodomain and WD repeat domain containing 3 |
| *C. elegans* | *Caenorhabditis elegans* |
| *C. pipiens* | *Culex pipiens* |
| C3 | Complement component 3 |
| CCR4 | C-C Motif Chemokine Receptor 4 |
| $CD4^+$ | Cluster difference 4 |
| Cdc42 | Cell division cycle 42 |
| cDNA | Copy DNA |
| Cecrpoin | CEC |
| CED | Cell death protein |
| CFUs | Colony forming units |
| CHS1 | Chitin synthase enzyme 1 |
| CI | Cytoplasmic incompatibility |
| CLIPs | Clip domain serine proteases |
| CM | Conditioned medium |
| CNOT | CCR4-NOT transcription subunit complex |
| CRD | Carbohydrate-recognition domain |
| CRISPR-Cas9 | Clustered Regularly Interspaced Short Palindromic Repeats- associated protein 9 |
| cSPHs | Serine proteinase homologs |

| | |
|---|---|
| cSPs | Clip domain containing serine proteinases |
| CT method | Computed tomography |
| CTLs | C-type lectins |
| CuZnSOD2 | Super oxide dismutase 2 |
| *D. melanogaster* | *Drosophila melanogaster* |
| DAP-type | Diaminopimelic acid-type peptidoglycan |
| DCE | Dopachrome conversion enzyme |
| DDC | Dopa decarboxylase |
| DENV | Dengue virus |
| DETs | Differentially expressed transcripts |
| DHI | 5,6-dihyroxyindole |
| Dif | Dorsal-related immunity factor |
| dkd | Double knockdown |
| Dm | *Drosophila melanogaster* |
| Dnrl | Defense repressor 1 |
| Dome | Domeless |
| Dopa | Dihydroxyphenylalanine |
| Dpp | TGFβ homologue Decapentaplegic |
| DREDD | Death-related ced-3/Nedd2-like protein |
| DSB | DNA double strand breaks |
| Dscam | Down syndrome cell adhesion molecule gene |
| dsRNA | double stranded RNA |
| Duox | Dual oxidase 2 |

| | |
|---|---|
| DVRFs | Infection-responsive dengue virus restriction factors |
| *E. coli* | *Escherichia coli* |
| EGFR | Epidermal growth factor receptor |
| EM | Embryonic mortality |
| FADD | Fas-associated protein with death domain |
| FBNs | Fibrinogen domain immunolectin |
| FN3Ds | Fibronectin domain proteins |
| FokI | Enzyme naturally found in Flavobacterium okeanokoites |
| FOXO | Forkhead transcription factors |
| Fz2 | Frizzled-2 |
| GALE | Gal-lectin family |
| GAM | Gambicin |
| GFAT | Glucosamine-fructose-6-phosphate aminotransferase |
| GFP | Green fluorescent protein |
| GNBPs | Gram-negative bacteria binding proteins |
| GO | Gene ontology |
| GPI | Glycosylphosphatidylinositol |
| Gr | Gustatory receptor |
| $H_2O_2$ | Hydrogen peroxidase |
| HDF | Hemocyte Differentiation Factor |

| | |
|---|---|
| HdMv | Hemocyte-derived microvesicles |
| HEGs | Homing endonuclease gene |
| *hep* | Hemipterous |
| Hop | Hopscotch |
| HP | Hemolymph Proteinase |
| HPX2 | Heme peroxidase 2 |
| Hz | Hemozoin |
| I-PpoI | Intron-encoded endonuclease I from *Physarum polycephalum* |
| Iap2 | Inhibitor of apoptosis 2 |
| IGALE20 | Infection-responsive galactose lectin |
| IgSF | Immunoglobulin gene superfamily |
| IIT | Incompatible insect technique |
| IKK | IκB kinase |
| Imd pathway | Immune deficiency pathway |
| IMPer | Immunomodulatory peroxidase |
| IRC | Immune-regulated catalase |
| IRID | Infection responsive immunoglobulin domain |
| IRS | Indoor residual spraying |
| ISC | Intestinal stem cells |
| ISP13 | Immune-related serine protease 13 |
| ISPL5 | Immune-related serine protease-like sequence 5 |
| iTEP | Insect thioester-containing protein |

| | |
|---|---|
| ITNs | Insecticide-treated mosquito nets |
| IVM | Integrated control management |
| JAK/STAT | Janus kinase/signal transducers and activators of transcription |
| JNK | c-Jun N-terminal kinase |
| K63 | Lysine 63 |
| kd | Knockdown |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| Ken | Ken & barbie |
| LB | Luria Bertani |
| LD | Lipid droplets |
| LLINs | Long-lasting insecticidal nets |
| LOS | Lipooligosaccharide |
| LPS | Lipopolysaccharide |
| LRIM1 | Leucine-Rich Immune Molecule 1 |
| LRP1 | LDL receptor-related protein 1 |
| LRRD | Leucine-rich repeat domain protein |
| LRRs | Leucine-rich repeats |
| LSM | Larval source management |
| LTA | Lipoteichoic acid |
| Lys-1, lys-7, and lys-8 | Lysozyme genes |
| Lys-PGN | Lysine peptidoglycan |
| *M. luteus* | *Micrococcus luteus* |

| | |
|---|---|
| *M. sexta* | *Manduca sexta* |
| MAPK | Mitogen-activated protein kinase |
| MC1 | Mitochondrial carrier 1 |
| Mcr | Macroglobulin complement-related |
| MDL | MD2-like protein family |
| MEDEA | Maternal-effect dominant embryonic arrest |
| MIP | Melanization-inhibiting protein |
| miRNA | microRNA |
| ModSP | Modular serine protease |
| MP1 and MP2 | Melanization proteases |
| mRNA | messenger RNA |
| MyD88 | Myeloid differentiation primary response 88 |
| *N. crassa* | *Neurospora crassa* |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NOT | Negative on TATA-less |
| NOX5 | NADPH oxidase 5 |
| OD | Optical density |
| OTUs | Operational taxonomic units |
| OXPHOS | Oxidative phosphorylation |
| OXR1 | Oxidation Resistance 1 |

| | |
|---|---|
| *P. agglomerans* | *Pantoea agglomerans* |
| *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* |
| p.i. | Post infection |
| PAH | Phenylalanine hydroxylase |
| PAMPs | Pathogen-associated molecular patterns |
| PAP | Prophenoloxidase activating proteinase |
| pBM | Post blood meal |
| PBS | Phosphate buffered saline |
| Pbs21 | Surface protein of *Plasmodium berghei* |
| PCR | Polymerase chain reaction |
| PfGPIs | *Plasmodium falciparum* Glycosylphosphatidylinositols |
| *Pfs47* | *P. falciparum* 47 |
| PGE2 | Prostaglandin E2 |
| PGNs | Peptidoglycans |
| PGRP-L | PGRP Long |
| PGRPs | Peptidoglycans recognition proteins |

| | |
|---|---|
| PGRPS | PGRP Short |
| pH | Potential of hydrogen |
| PIAS | Protein inhibitors of activated STATs |
| PM | Peritrophic matrix |
| proHP | HP zymogen |
| proPAP-2 | PPO activating proteinase-2 precursor |
| proPO | Prophenoloxidase |
| proSAE | SPE-activating enzyme zymogen |
| proSPE | SPE zymogen |
| proSPH | SPH precursor |
| proSpz | Spätzle proprotein |
| PRRs | Pattern recognition receptors |
| Psh | Persephone |
| PTP61F | Protein tyrosine phosphatase |
| Puc | Puckered |
| qRT | quantitative Real Time |
| RanBP3 | Ras-like guanine nucleotide-binding protein 3 |
| Rel1 | Relish1 |
| Rel2 | Relish2 |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RNA-seq | RNA sequencing |
| RNS | Reactive nitrogen species |

| | |
|---|---|
| ROS | Reactive oxygen species |
| *S. aureus* | *Staphylococcus aureus* |
| *S. marcescens* | *Serratia marcescens* |
| SAE | SPE-activating enzyme |
| Sax | Saxophone |
| SCRB | Scavenger receptors |
| SIT | Sterile insect technique |
| SLC25 | Solute carrier family 25 |
| Sm | *Serratia marcescens* |
| SM1 | Salivary gland and midgut peptide 1 |
| SNP | Single-nucleotide polymorphism |
| SOCS | Suppressor of cytokine signaling |
| SP | Serine protease |
| SPE | Spatzle processing enzyme |
| SRPN/Spn | Serpin |
| Spz | Spatzle |
| Tab2/Tak1 | TAK1-binding protein 2/TGF β -activated kinase 1 |
| TALE protein | Transcription activator-like effector protein |
| TALENs | Transcription activator-like effector nuclease |
| TEP1 | Thioester containing protein 1 |
| TEs | Transposable elements |
| TH | Tyrosine hydroxylase |

| | |
|---|---|
| TIR | Toll/IL-1R homologous region |
| Tm | *Tenebrio molitor* |
| Tm-mas | *Tenebrio* masquerade-like serine proteinase homologue |
| TNF-α | Tumor necrosis factor alpha |
| Ubc13 | Bendless |
| Ubc5 | Effete |
| UEV1a | Ubiquitin–conjugating enzyme |
| Upd | Unpaired |
| Vps 60 | Vacuolar protein sorting 60 |
| WASP | Wiskott–Aldrich syndrome protein |
| WHO | World Health Organization |
| α-2Ms | alpha-2 macroglobulins |

# CHAPTER I

# INTRODUCTION

## A. Malaria

### *1. Global status of the disease*

Malaria is a disease caused by parasites of the genus *Plasmodium* that are transmitted to humans through the bite of infected female mosquitoes of the genus *Anopheles.* Malaria has plagued humankind throughout history. In fact, the disease is traced back to at least 4,000 years ago, as evident from the identification of ancient DNA (aDNA) of the major human malaria parasite, *Plasmodium falciparum* (*P. falciparum*), in ancient Egyptian mummy tissues [1]. According to the World Health Organization (WHO), an estimated 219 million clinical cases of malaria and 405000 deaths from the disease were reported worldwide in 2018. The disease continues to strike hardest against pregnant women and children aged under 5 years, with the latter accounting for 67% of all malaria deaths worldwide. The burden has been heaviest in the WHO African Region which accounts for approximately 94% of all malaria deaths. According to WHO, there exists currently two core interventions in the fight against malaria: vector control and chemoprevention. However, the emergence of parasite resistance to antimalarial drugs [2] and of mosquito resistance to insecticides [3], have hindered global efforts in reducing the burden malaria.

### *2. Plasmodium life cycle in Anopheles mosquitoes*

Malaria is caused by a unicellular parasite ingested by a female mosquito through blood feeding on an infected individual. Forty-one species of mosquitoes belonging to the genus *Anopheles* are capable of transmitting malaria at a level that constitutes a major concern to public health [4]. Five *Plasmodium* species including *P. falciparum*, *Plasmodium malariae* (*P. malariae*), *Plasmodium ovale* (*P. ovale*), *Plasmodium vivax* (*P. vivax*) and *Plasmodium knowlesi* (*P. knowlesi*) are recognized as causative agents of human malaria [5]. As they shuttle between the mammalian host and mosquito vector, malaria parasites go through different stages of differentiation associated with distinct gene expression programs [6-8] and cell morphologies (Fig. 1). In the vector, the parasite life cycle starts when an *Anopheles* female mosquito ingests male (microgametocytes) and female (macrogametocytes) gametocytes during a blood meal. Exflagellation of gametocytes followed by fertilization and formation of a zygote are triggered in the mosquito midgut by the presence of xanthurenic acid and a concomitant decrease in temperature and increase in pH [9, 10]. The zygote differentiates into a motile and elongated ookinete, which penetrates the peritrophic matrix surrounding the blood bolus and invades the midgut epithelium at approximately 24 hrs after blood feeding. The ookinete emerges from the basal side of the midgut epithelium facing the haemocoel, where it lodges beneath the basal lamina and differentiates into a sessile oocyst. Oocysts grow in size and undergo sporogony to produce thousands of sporozoites over a period of two weeks. Afterwards, mature sporozoites are released into the hemolymph from where they invade the salivary glands. The delivery of sporozoites with the mosquito saliva into a new host marks the end of the cycle. Malaria parasites undergo severe bottlenecks during their development

in the mosquito vector, specifically at the ookinete and pre-ookinete stages due to several factors pertaining to the mosquito and the human host. Human factors ingested with the blood meal including, cytokines, reactive nitrogen species (RNS), white blood cells, and complement proteins have been shown to remain active in the mosquito midgut for several hours after blood ingestion [11-13]. RNS production in the midgut maybe triggered by the action of tumor necrosis factor alpha (TNF-α) on white blood cells. TNF- $\alpha$ , through the RNS-mediated reduction of exflagellating males, was shown to reduce the formation of *Plasmodium berghei* (*P. berghei*) ookinetes [14]. The parasite is also susceptible to complement attack, with late zygotes and ookinetes being less protected than early stages (gametocytes, gametes and early zygotes) [12, 13, 15, 16]. In the mosquito, components of the innate immune system, which will be detailed in subsequent sections of the thesis, as well as the microbiota have a dramatic effect on the survival of *Plasmodium* parasites in the midgut. Ookinetes that survive the midgut lumen environment and eventually invade the midgut epithelial barrier are exposed to two phases of the mosquito innate immune response. Early-phase immunity limits ookinete survival or the ookinete to oocyst transition, whereas “late-phase" immunity limits oocyst survival [17].

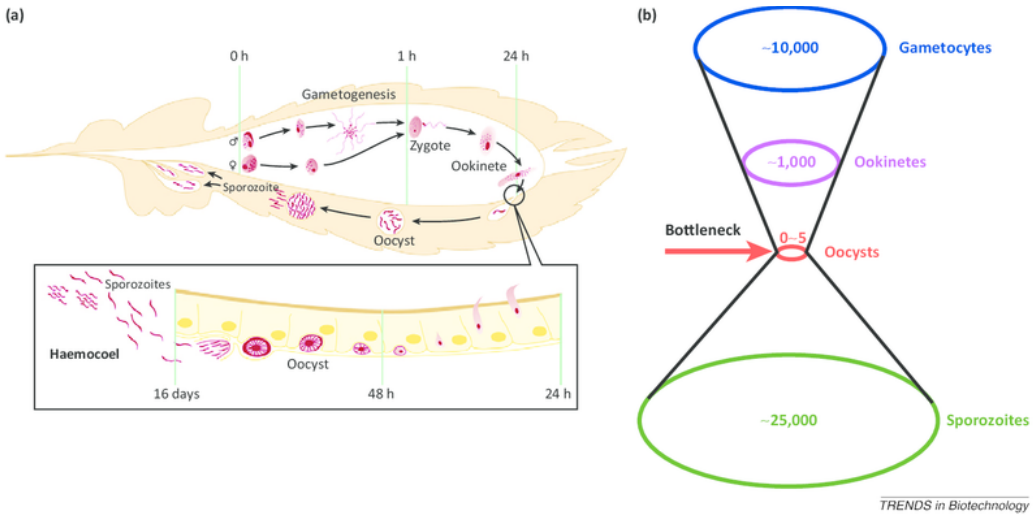


Figure 1. The malaria parasite life cycle in the mosquito vector.

**(a)** *Plasmodium* life cycle in the mosquito. The developmental time corresponding to each stage of the model rodent malaria parasite *P. berghei* (maintained at 20°C) is indicated. **(b)** In the mosquito midgut, a severe bottleneck occurs in the numbers of *Plasmodium* parasite at the ookinete to oocyst transition. Adapted from [18]

### *3. Vector-based control strategies*

Malaria remains a global public health challenge in the twenty-first century. Several control strategies are currently used to roll back the disease while several others are still under investigation. In the following sections, we provide a synopsis of these different strategies.

a. Insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS):

Perhaps one of the key malaria prevention and control interventions are insecticide-treated mosquito nets (ITNs). ITNs include long-lasting insecticidal nets (LLINs) lasting up to 3 years, and conventionally treated nets, where the insecticide is active for

up to 12 months [19]. Indeed, ITNs have proven their efficiency in reducing the disease burden [20, 21]; however, shortfalls and inequities exist among which is the mere possession of ITNs that has been shown to be associated with factors such as proximity to distribution sites, cost, socioeconomic status, and the method of distribution. Another major issue is the resistance to the pyrethroid insecticides used in ITNs, the expansion of which could jeopardize the current momentum of reduction and elimination of malaria [22, 23].

Indoor residual spraying (IRS) is another vector control strategy that involves spraying internal walls and ceilings of dwellings using insecticides with residual action. IRS effectiveness is proven and reported in several studies [24-26] despite concerns about its long-term sustainability [27] and the high risk associated with the use of IRS on human health and the environment.

b. Larval source management:

Perhaps one of the oldest and often dismissed interventions in the fight against malaria is larval source management (LSM) that focuses on disrupting potential breeding sites of mosquitoes in the aim to prevent completion of their aquatic life cycle. This can be accomplished through habitat modification, habitat manipulation, biological control through introduction of natural enemies into aquatic habitats, and most commonly through larvicidal applications [19].

Indeed, a promising approach in the fight against malaria is integrated vector control management (IVM) that combines several vector control strategies as a means to optimize efficacy [28, 29].

c. Sterile Insect Technique:

A promising genetic control strategy of mosquito vectors is the sterile insect technique (SIT), which is a species-specific environmentally non-polluting method that involves releasing large numbers of sterile males in an attempt to decrease native female reproductive potential, ultimately suppressing the native population [30-33]. There are several approaches for inducing sterility including, chemosterilization, hybrid sterility that relies on mating two close species, and the more commonly used sterilization by irradiation [34]. The latter relies mostly on the application of ionizing radiation that induces chromosomal aberrations in exposed germ cells; Sperm and ova are viable but carry dominant lethal mutations [35]. Indeed, SIT has proven successful in eradicating three target pests: the North and Central American screwworm fly from southern United States, Mexico, and all of Central America [36-38], the Mediterranean fruit fly from Central America and Mexico [39] and the tsetse fly from the island of Zanzibar in Tanzania [33, 40, 41]. However, a major challenge is the development of an accurate sex separation method, crucial for the required male-only release that can be applied to a large scale [42]. The difficulty of irradiating males without reducing their mating competitiveness and survival is yet another challenge of this technique [43-45]. Transgenic sterility is another method that causes sterility by targeting pro-apoptotic genes or the X chromosome leading to early developmental arrest. Again, a direct effect of this technology is potential reduction in fitness [34]. A promising alternative is the incompatible insect technique (IIT) that makes advantage of the natural phenomenon of cytoplasmic incompatibility (CI) induced by the Gram-negative bacterium *Wolbachia*. In IIT, males are infected with the maternally inherited endosymbiotic bacteria *Wolbachia* and released into the field allowing mating with females that are not

infected with the same *Wolbachia* strain, resulting in embryonic mortality (EM) [46, 47]. However, biological, i.e., competitive exclusion with other bacteria in the gonads such as *Asaia* [48-50] and technical, i.e., egg microinjection difficulties, have hampered the progress in transferring *Wolbachia* between mosquito species. In fact, only one stable transfected *Wolbachia* colony has been described in *Anopheles stephensi* (*A. stephensi*) [51].

d. Paratransgenesis:

The midgut of *Anopheles* mosquitoes harbors a highly diverse microbiota, among which are members of five dominant phyla *Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria,* and *Firmicutes* [52]. Paratransgenesis is a “Trojan Horse" strategy that interferes with the vector’s ability to transmit disease by introducing into the microbiota engineered symbiotic microbes that produce anti-*Plasmodium* effector molecules [53]. This approach takes advantage of the fact that the microbiota resides in the same compartment where the development of ookinetes, the most vulnerable stages of the malaria parasite, occurs [54]. Furthermore, following a blood meal, members of the midgut microbiota increase in number [55], triggering a concomitant increase in anti-*Plasmodium* effector molecules produced by the genetically modified bacteria. Effector molecules in question may have multiple modes of action: Direct parasite killing, interaction with parasite surface proteins required for midgut invasion, interaction with target proteins on mosquito midgut or salivary gland epithelia, and manipulation of mosquito immune system [56]. For instance, feeding mosquitoes prior to an infective blood meal on a transgenic *Escherichia coli* (*E. coli*) strain genetically engineered to display two anti-*Plasmodium* effector molecules, salivary gland and midgut peptide 1 (SM1) and phospholipase-(A)2, on their outer membrane significantly

inhibited *P. berghei* development [57]. In a separate study, a natural symbiotic bacterium *Pantoea agglomerans* (*P. agglomerans*) was engineered to produce and secrete a variety of anti-*Plasmodium* effector proteins in the mosquito midgut. Interestingly, the engineered *P. agglomerans* strains significantly inhibited development of *P. falciparum* and *P. berghei* [58]. Wang *et al.* reported that the use of a species of *Serratia* (strain AS1) engineered to secrete different anti-*Plasmodium* effector proteins, compromised *P. falciparum* oocyst development in the midgut [59]. Recently, the midgut symbiont *Asaia* engineered to express the antiplasmodial protein scorpine in a blood-meal inducible manner was shown to significantly inhibit *P. berghei* ookinete development in *A. stephensi* [60]. Other than reducing vector competence, engineered bacteria can act through causing pathogenic effects in the host or through interfering with reproduction or embryogenesis [61].

e. Gene editing and gene drive

Another promising approach which is currently under investigation and laboratory testing is combining gene editing with gene drive to manipulate the mosquito population in ways that reduce malaria transmission. Indeed, genetic engineering is a technology that may have sounded like science fiction at the beginning but has advanced greatly during the past years. In general, two main approaches have been proposed to genetically manipulate mosquitoes in order to control malaria: Population suppression that aims at reducing mosquito numbers, and population modification that hinders the vector's ability to support parasite development. Proof of principle studies of both approaches have been reported. Regarding the former approach, Galizi *et al.* generated a synthetic sex distortion system that exploits I-PpoI, a homing endonuclease that is able to cleave ribosomal gene sequences located exclusively on the mosquito's X

chromosome, hence, preventing it from being transmitted to the next generation, and resulting in fully fertile mosquito strains that produce >95% male offspring [62]. In a similar context, Hammond *et al.* identified three genes that confer a recessive female-sterility phenotype upon disruption, and inserted into each locus Clustered Regularly Interspaced Short Palindromic Repeats- CRISPR associated protein 9 (CRISPR-Cas9) gene drive constructs designed to target and edit each gene [63]. As for the latter approach, the concept was tested for the first time by genetically modifying *A. stephensi* for midgut expression of the SM1 peptide that strongly inhibits ookinete midgut invasion through binding to a putative ookinete receptor on the luminal surface of the midgut epithelium [64]. Indeed, the genetically engineered mosquitoes were substantially impaired in their ability to transmit the parasite [65]. Subsequent reports from different laboratories utilizing a variety of effector molecules reached a similar conclusion: it is possible to reduce *Plasmodium* transmission via genetic modification of the vector mosquito [66-71]. Most recently, transgenic mosquitoes expressing multiple endogenous and exogenous effectors targeting multiple stages of the parasite exhibited strong suppression of *P. falciparum* in *A. stephensi* [72].

A crucial challenge in genetic engineering approaches is to drive anti-malaria effector genes into wild mosquito populations. Several gene drive systems exist including, the use of transposable elements, nuclease-based systems (Homing endonuclease genes (HEGs), Transcription activator-like effector nucleases (TALENs) and CRISPR), microorganisms such as *Wolbachia*, and the toxin-antidote system such as MEDEA (maternal-effect dominant embryonic arrest) [73, 74]. Transposable elements (TEs) are mobile genetic elements that have the ability to replicate and spread in a genome. According to their transposition mechanisms, TEs can be categorized as

being either ribonucleic acid (RNA)-mediated (Class I) that involve an RNA intermediate or direct DNA-mediated (Class II) elements [75]. In addition to their use as vectors to insert genes into the genome of a species to change its genetic makeup, TEs can also be used for gene trapping, enhancer trapping, and genome-wide insertional mutagenesis studies [76-78]. In mosquitoes, DNA transposons such as *Hermes, MosI, minos*, and *piggyBac*, have demonstrated varied degrees of utility as transformation tools [79]. HEGs are a class of simple selfish genetic elements that could also be exploited for the purpose of spreading a genetic modification from laboratory mosquitoes to field populations. HEGs encode highly specific endonucleases that can induce DNA double strand breaks (DSB) and fool an organism's recombinational repair system into copying the HEG across onto the other chromosome in a process referred to as "Homing" [80]. Another class of nucleases useful for genome editing comprises TALENS. These nucleases consist of a FokI nuclease domain linked to a DNA recognition domain that comes from a transcription activator-like effector protein (TALE) protein. TALE proteins are known to be injected by invading bacteria of the genus *Xanthomonas* into plant cells where they enter the nucleus, bind DNA and activate transcription of host genes needed for growth and division of the bacteria [81]. The development of bacterial adaptive immune system which includes, CRISPR and CRISPR/Cas followed the engineering of TALENs, and since then has revolutionized the process of genome engineering [82]. The CRISPR system is based upon an endonuclease, called Cas9, directed by a short guide RNA to its genomic target. It is a simple yet powerful tool that allows researchers to target and cut almost any site in the genome. Several groups demonstrated CRISPR-based homing in yeast and fruit flies before gene drives built for population replacement and population suppression were

demonstrated in *A. stephensi* and *Anopheles gambiae* (*A. gambiae*), respectively [63, 83-86]. Another example of a yet less invasive gene drive system is *Wolbachia*, a diverse group of maternally inherited intracellular bacteria present in arthropods [87-89]. Cytoplasmic incompatibility (CI), perhaps the most widespread and prominent feature that *Wolbachia* endosymbionts impose on their hosts [90, 91], results in EM in matings between insects of the same species harboring different *Wolbachia* strains [92, 93]. In other words, eggs of uninfected females cannot be fertilized in matings with males infected with *Wolbachia*, but eggs of infected females can be fertilized by uninfected males or males infected with the same Wolbachia strain, giving infected females a fitness advantage. This allows for an increase in the frequency of *Wolbachia* and any desirable traits associated with them over multiple generations [94]. Similarly, a *Medea* system rapidly spreads itself, and any linked cargo genes, through a target population. *Medea* is a selfish gene that encodes a toxin–antidote combination composed of a microRNA (miRNA) toxin that is expressed during oogenesis in *Medea*-bearing mothers, and a tightly linked antidote expressed early during embryogenesis in *Medea*-bearing progeny. A *Medea*-bearing mother would express the toxin in her germline, resulting in suppression of an essential embryonic gene and ultimate disruption of normal development during embryogenesis. Only offspring that inherit *Medea* receive a tightly linked antidote, consisting of a miRNA-resistant copy of the targeted essential gene that allows for restoration of normal development [95].

## B. Molecular and cellular basis of mosquito immunity

Mosquitoes often acquire pathogens through feeding on nectar, blood feeding or through breaks in their cuticle following a physical injury or pathogen-driven

degradation. Intricate co-evolutionary processes between mosquitoes and pathogens determine the host's resistance or susceptibility to infection [96]. Lacking an adaptive immune system that in vertebrates relies on a vast repertoire of antibodies or variable lymphocyte receptors, insects rely solely on innate immune responses in their defense against invading pathogens [97, 98]. Mosquitoes possess a complex and effective immune system that can be classically divided into cellular and humoral responses that work in concert to orchestrate an efficient and rapid response against invaders. In several instances, there is a fine line between the two arms as many humoral components are produced by hemocytes [99-104], and some humoral molecules participate in cellular immunity (for example the role of the thioester containing protein 1 (TEP1) as an opsonin, discussed in later sections). In mosquitoes, the cellular effector arm of the immune system consists mainly of professional immune cells called hemocytes which mediate responses including, phagocytosis, encapsulation and nodulation [105]; whereas humoral effector responses include antimicrobial peptide production by fat body and epithelial cells, components of the phenoloxidase cascade responsible for melanization, the production of reactive oxygen and nitrogen intermediates, and complement mediated attack [106] (Fig. 2).

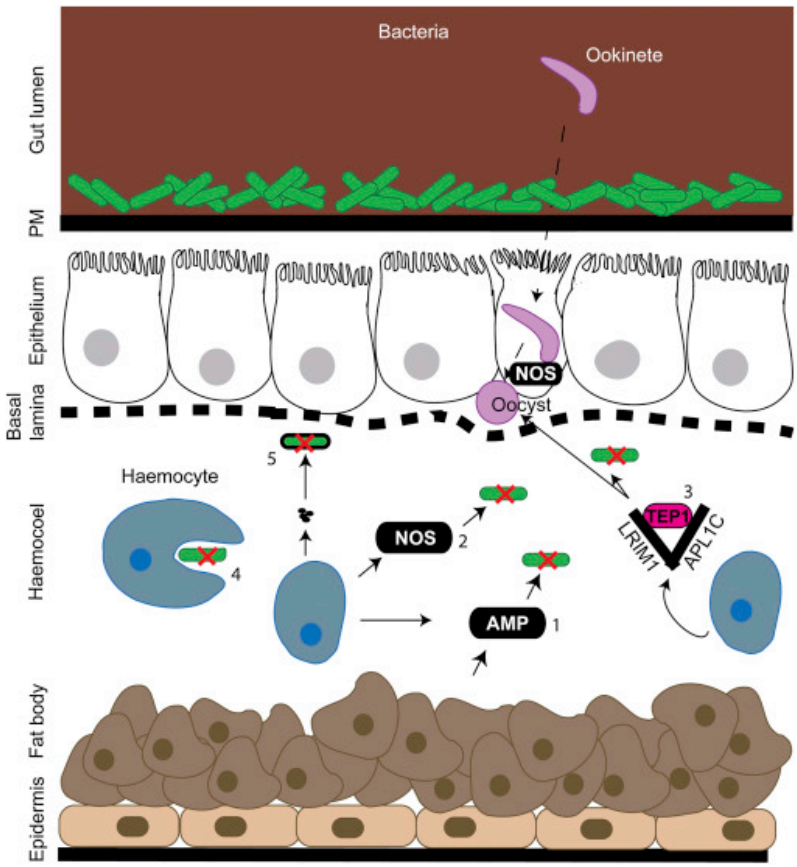


Figure 2. Mosquitoes mount an efficient immune response against infectious agents. Antimicrobial peptides (AMPs) produced by the fat body, hemocytes and epithelial cells cause pathogen lysis. Nitric oxide synthase (NOS) transcription is induced in epithelial cells by invading *Plasmodium* ookinetes and in hemocytes by systemic bacterial infections. A complement-like protein (TEP1) associated with leucine-rich proteins (APL1C and LRIM1) are secreted by hemocytes and deposited on the surface of *Plasmodium* parasites, bacteria and fungi causing their lysis or melanization. Pathogens are phagocytosed by granulocytes. A prophenoloxidase cascade results in melanin deposition on pathogen surfaces. (Adapted from [107])

### *1. Pathogen recognition*

On a molecular basis, immunity is initiated upon recognition of "non-self" entities so-called pathogen-associated molecular patterns (PAMPs) by pattern

recognition receptors (PRRs) [108, 109]. PAMPs include, among others, β-1,3-glucans which constitute part of the fungal cell wall and lipopolysaccharides (LPS) and peptidoglycans (PGN) which are components of bacterial cell surfaces [110]. Various PRRs have been identified and isolated from invertebrates [111-114]. Following recognition, some PRRs can directly invoke cellular effector mechanisms such as encapsulation and phagocytosis, others trigger activation of intracellular immune signaling pathways that control transcription of immune effector genes, while a group of PRRs can activate both, humoral effector responses in the hemolymph, such as melanization, and signaling pathways in immune tissue cells [115, 116].

In the following sections below, we describe briefly the major PRRs families whose members exhibited clear roles in the mosquito anti-microbial defense based on functional genetic studies, and which include, the peptidoglycan recognition proteins (PGRPs), the Gram-negative bacteria binding proteins (GNBPs), fibrinogen domain immunolectin (FBN), the immunoglobulin superfamily (IgSF), and the C-type lectins (CTLs) [117].

a. Peptidoglycan recognition proteins (PGRPs):

First discovered in two Lepidopteran insects [118, 119] and then in many other insects as well as in higher animals including humans, PGRPs recognize and primarily bind to peptidoglycan, an essential component of bacterial cell walls [120, 121]. PGRPs are classified into two subfamilies, short (S) which are usually secretory proteins, and long (L) which are mostly intracellular or membrane-bound proteins [122]. Among the seven putative PGRPs identified in *A. gambiae* mosquitoes [123], four belong to the long subfamily ( PGRP-LA, -LB, -LC and -LD) and three to the short subfamily

(PGRP-S1, -S2 and –S3). Among these, PGRPLB, PGRPS2, and PGRPS3 are putative amidases while PGRPLD, PGRPLC, PGRPS1, and PGRPLA are considered non-catalytic [123]. PGRPLC seems to be central to antibacterial defense [124]. Silencing *PGRPLC* was shown to increase infection by *P. falciparum* thereby emphasizing the role of the immune deficiency (Imd) pathway in *A. gambiae* immunity against *Plasmodium*. It is worthmentioning that this effect of PGRPLC on *Plasmodium* survival is directly related to the bacteria residing in the mosquito midgut [124]. PGRPLB seems to be involved in anti-*Plasmodium* defense as evident from its elevated expression following *Plasmodium* infection of adult mosquitoes [125], which persists throughout the parasite life cycle in the vector [123]. Genetic analysis by RNA interference (RNAi) revealed that PGRPLB enhances mosquito tolerance to *Plasmodium* infection [126]. The same study showed that each of PGRPLA and PGRPS2/S3 act as *Plasmodium* antagonists, and that following a blood meal, PGRPLA1 and PGRPLB positively and negatively regulate the activation of the Imd pathway, respectively. In fact, PGRPLB seems to play a dual role in *Anopheles* mosquitoes, facilitating parasite infection and protecting natural gut bacteria [126, 127]. In *A. stephensi*, PGRP-LD limits *P. berghei* infection by maintaining gut homeostasis and in turn the structural integrity of the peritrophic matrix (PM) [128].

b. Gram-negative bacteria-binding proteins (GNBPBs):

*A. gambiae* GNBPs comprise six members belonging to two subfamilies; Subfamily A includes, GNBPA1 and GNBPA2, while subfamily B includes, GNBPB1, GNBPB2, GNBPB3, and GNBPB4 [123, 129]. GNBPs are alternatively called beta-glucan-binding proteins (β-GBPs) because they contain a conserved β-1,3-glucan

binding domain that was initially shown to bind fungal cell walls [130-132]. With the exception of GNBPA1, all members of the *A. gambiae GNBP* gene family possess a signal peptide sequence indicating that they are secreted [133]. Moreover, *A. gambiae GNBPs* showed a tissue specific and infection responsive expression. Whereas challenging with *E. coli* caused an induction of all six *GNBP* genes, *Staphylococcus aureus* (*S.aureus*) challenge induced only *GNBPA1* and *GNBPB4*. Survival studies identified several GNBPs required for mosquitoes to tolerate bacterial infections. For instance, silencing any of *GNBPA2*, *B1*, *B3*, and *B4* reduced mosquito tolerance to *E. coli* infections, while silencing of *GNBPB4* compromised survival after challenge with *S. aureus.* The authors further revealed that certain GNBPs exhibited *Plasmodium*-species specific effects; For instance, silencing *GNBPA2* reduced resistance to *P. falciparum* infection, whereas silencing *GNBPB3* and *GNBPB4* reduced resistance to *P. berghei* infection [133]. Altogether, these data indicate a broad role of GNBPs in mosquito immunity.

c. Fibrinogen Domain proteins (FREPs):

An important immune gene family that is evolutionary conserved from invertebrates to mammals is the fibrinogen-related protein (FREPs) family also known as FBN family [134-137]. The *FREP* gene family is the largest immune and pattern recognition gene family in *A. gambiae*, with 59 putative members [138]. FBNs are immune-responsive to challenge with bacteria and *Plasmodium*. Dong *et al.* showed that FBN8 and FBN9 strongly influence both *P. falciparum* and *P. berghei* development. Christophides *et al.* also demonstrated that *FBN9* was strongly inducible both by bacteria and during *Plasmodium* penetration of the midgut [123]. Interestingly, it seems that FBN9 activity requires direct contact with pathogen surfaces as evident from its co-

localization with both rodent and human malaria parasites in the mosquito midgut epithelium and the fact that it forms dimers that bind Gram positive and negative bacterial surfaces with variable affinities [138]. This broad spectrum of activity against multiple types of pathogens reflects an important and more general role in the innate immune system and is not restricted to FBN9. FBN39 is another FBN that demonstrated even a more pronounced effect on *P. falciparum* development. Like *FBN9*, *FBN39* is also induced upon bacterial challenge with *E. coli* [139]. Silencing *FBN8, 9*, or *39* increased mosquito susceptibility to *P. falciparum,* while silencing *FBN6* and *9* increased susceptibility to *P. berghei*. Silencing *FBN5* or *FBN26* had no effect on infection. In addition, gene silencing of members of the FBN family significantly altered mosquito tolerance to *E. coli* (*FBN4, 5, 6, 9,* and *22*) and *S. aureus* (*FBN4, 5, 6,* and *26*) infections as well as resistance to four Gram-negative bacterial species: *Serratia*, *Asia bogorensis*, *Pseudomonas veronii*, and *Sphingomonas* (*FBN 39* and *22*). *Simões et.al* developed FBN9 immunolectin overexpressing transgenic mosquitoes that are more resistant to both Gram positive and negative bacteria and to *P. berghei* but not *P. falciparum* pointing to species-specific effects of this gene [140].

d. <u>The immunoglobulin superfamily (IgSF) proteins:</u>

The *A. gambiae* transcriptome encodes 138 proteins with at least one immunoglobulin domain. Garver *et al.* showed that overall 85 *IgSF* genes were differentially regulated upon challenge with *Plasmodium*, Gram-negative or Gram-positive bacteria. Functional characterization of infection responsive immunoglobulin domain (*IRID*) genes revealed that *IRID3, IRID5* and *IRID6* are required for mosquito survival to bacterial infections, while *IRID4* and *IRID6* are implicated in anti- *Plasmodium* defense [141]. In addition to IRIDs, splice variants of the *Anopheles*

*gambiae* Down syndrome cell adhesion molecule gene (*AgDscam*) are also involved in mosquito immunity. Dong *et al.* showed that infection-responsive alternative splicing of the hypervariable immunoglobulin domain containing receptor *AgDscam* generates a broad range of PRRs implicated in immune defense in the malaria vector *A. gambiae*. In addition, AgDscam is a determinant of tolerance and resistance to bacterial infections and is implicated in defense against the rodent malaria parasite. AgDscam also mediates phagocytosis of bacteria with which it associates in a splice form–specific manner [142]. In a subsequent study, it was shown that AgDscam suppresses *P. falciparum* development and interacts with the surface of ookinete stage parasites in the mosquito midgut epithelium. Moreover, the authors showed that by regulating the alternative splicing of *AgDscam*, the Imd and Toll pathways mediate AgDscam-mediated species-specific defenses against *Plasmodium* [143]. Collectively, the two studies showed that the single germ-line encoded gene can produce different splice form repertoires in response to challenge with each of at least eight different immune elicitors, ultimately providing the mosquito with a remarkable flexibility in pathogen recognition. *AgDscam* splice-form diversity is not restricted to laboratory studies, as Smith *et al.* reported significant increases in *A. gambiae* Dscam receptor diversity in parasite-exposed mosquitoes [144].

e. C-type lectins (CTLs):

C-type lectins are a large lectin family in animals whose members bind to carbohydrates in a calcium-dependent manner through the carbohydrate-recognition domain (CRD). CTLs function outside cells as secreted proteins or otherwise as membrane-bound proteins [145]. While some insect CTLs have a transmembrane domain, the majority possess signal peptides and function as secreted proteins [146].

The number of CTL genes varies greatly in different orders of insects as well as among genera of insects [129, 147-150]. CTLs play important roles in insect immune responses (Fig. 3), functioning as pattern recognition receptors involved in agglutination, opsonization, encapsulation and melanization of microbes [146]. In addition, certain insect CTLs interact with the microbiota to maintain gut microbiome homeostasis [151] and can be utilized by viruses to facilitate entry into host cells [152, 153]. Some CTLs possess unique functions such as stimulating hemocyte proliferation [154]. The *A. gambiae* genome includes 25 *CTL* genes classified into different subgroups based on the number of CRDs and domain architectures [148]. In *A. gambiae*, the expression of two *CTL* genes (*CTL4* and *CTLMA2*) was found to be induced by bacterial infections. *In vivo* RNAi analysis further revealed a role for these two CTLs in defense against Gram-negative, but not Gram-positive bacteria. CTL4 and CTLMA2 are secreted into the hemolymph in the form of an obligate disulfide-linked heterodimer [155]. These CTLs seem to have pleiotropic functions as they were shown to protect *P. berghei* ookinetes against the potent mosquito melanization response [156], hence acting as parasite agonists. This *Plasmodium* protective feature seems to be specifically utilized by the parasite for evasion of mosquito defense mechanisms as evident by the fact that silencing *CTL4* and *CTLMA2* did not affect Sephadex bead melanization [157]. Moreover, the agonistic effect of these CTLs on *P. berghei* development was not observed with *P. falciparum* infections in the same mosquito species and strain [158, 159], unless mosquitoes were infected with a higher gametocytemia of the human parasite, indicating that the RNAi phenotypes of *CTL4* and *CTLMA2* with respect to *P. falciparum* infections are dependent on infection intensity [159].

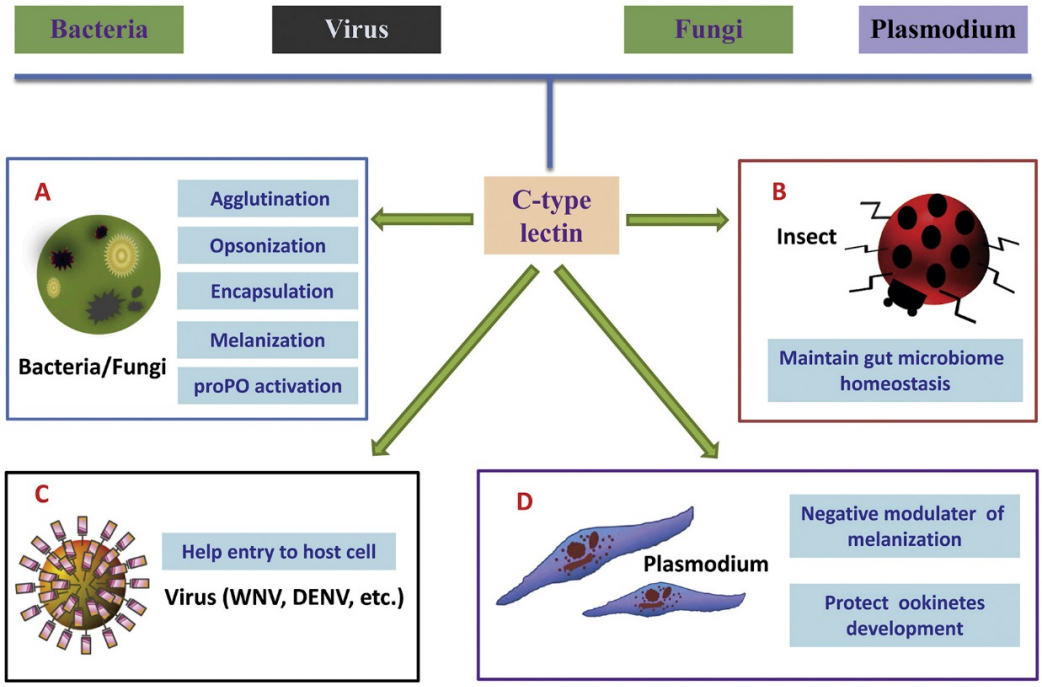


Figure 3. Proposed functions of insect C-type lectins.

Insect C-type lectins function as pattern recognition receptors involved in agglutination, opsonization, encapsulation, melanization of microorganisms and prophenoloxidase (proPO) activation **(A)**, maintain gut microbiome homeostasis through interaction with the microbiota **(B)**, are utilized by viruses to facilitate entry into host cells **(C)** and act as agonists protecting *Plasmodium* parasites from melanization **(D)**. (Adapted from [146])

### *2. Immune signaling pathways*

Following recognition of PAMPs by PRRs, highly complex intracellular signaling cascades are triggered to provide protection against a broad range of invaders. Toll, Imd and Janus kinase/signal transducers and activators of transcription (JAK/STAT) are regarded as the main pathways regulating innate immunity in insects. Signaling downstream of these pathways ultimately leads to induction of immune-related and other genes that collectively will determine the host resistance and tolerance to the invading microbe (Fig. 4).

a. Toll pathway:

The Toll pathway was initially identified in view of its essential role in the early embryonic development of *Drosophila melanogaster* (*D. melanogaster*) [160]. In *Drosophila*, the Toll pathway is induced by Gram-positive bacterial and fungal infections [161-167]. Signaling is initiated by hydrolysis of proSpätzle to Spätzle by spätzle processing enzyme (SPE) followed by the subsequent binding of the active form of Spätzle to the Toll receptor [111, 161, 168]. Briefly, two complex cascades link microbial recognition to SPE activation: the PRR and Persephone (Psh) pathways. The PRR pathway is initiated by binding of GNBP1 and PGRP-SA that sense Gram-positive bacteria and GNBP3 that senses fungi to their respective microbial ligands. Following recognition, an upstream serine protease (SP), modular serine protease (ModSP), is activated leading to the subsequent activation of Grass, followed by the maturation of SPE [111, 169-172]. In the Psh pathway, proteolytic activation of Psh by enzymes secreted by fungi [171] and Gram-positive bacteria [170] cleaves SPE triggering the Toll pathway (Figs. 9&10).Toll activation by Spatzle (Spz) triggers a cassette of proteins consisting of myeloid differentiation primary response 88 (MyD88), Tube, and Pelle, to assemble at the Toll/IL-1R homologous region (TIR) domain of Toll eventually leading to the phosphorylation and proteasome-dependent degradation of Cactus, an ankyrin protein that inhibits the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor Dorsal-related immunity factor (Dif). Dif then translocates into the nucleus to regulate transcription of hundreds of genes including those encoding anti-microbial peptides (AMPs) [112, 166, 173-177]. It is likely that the Toll pathway is also involved in cellular immunity, as mutants that

constitutively activate this pathway harbor over reactive blood cells that form melanotic capsules [178] similar to the cellular response to parasites [179].

Comparative genomic analysis of immunity-related genes revealed that most intracellular components of immune signaling pathways are well conserved between the fruit fly *D. melanogaster* and the malaria mosquito *A. gambiae*, but several differences exist [123]. It is well established that Relish1 (Rel1), previously known as Gambif1, a homologue of *Drosophila* Dorsal, is a downstream mediator of the mosquito Toll immune pathway [180] that is elicited by Gram-positive bacteria, fungi and *Plasmodium* [181]. Infection-responsive activation of this pathway ultimately leads to the release of the NF-κB transcription factor Rel1 from Cactus, followed by its nuclear translocation and transcriptional activation of immune effector genes such as AMPs, which in mosquitoes comprise defensins, cecropins, attacin and gambicin [182]. It has been shown that activation of the Toll pathway in *A. gambiae* by silencing Cactus, the negative regulator of Rel1, aborts development of the rodent malaria parasite *P. berghei* [183, 184]. Depletion of Cactus increases the basal expression levels of negative (*TEP1* and leucine-rich immune molecule 1 (*LRIM1*)) and positive (Serpin 2 (*SRPN2*) and *CTL4*) regulators of *P. berghei* development. These effects persist through *P. berghei* infection and boost the postinvasion response of ookinetes [183]. Cactus depletion was also shown to confer partial resistance to *P. falciparum* infections in a separate study [184]. A study investigating the effect of over activating the Toll pathway on the relative abundance of different *Anopheles* hemocyte populations revealed that the over activation of Toll signaling favors the differentiation of the oenocytoid lineage as evident from a concomitant reduction in prohemocytes when oenocytoids increase in response to *Cactus* silencing [185]. In addition, the

number of oocysts present was significantly lower in mosquitoes that received cell-free hemolymph from *Cactus*-silenced donors relative to the *dsLacZ* controls but transfer of cells had no effect on infection [185] suggesting that overactivation of Toll signaling results in the release of a soluble factor that mediates the enhanced antiplasmodial response observed when the suppressor Cactus is silenced [183, 184].

Transgenic alteration of Toll immune pathway through RNAi knockdown of AaRel1 in the female mosquito *Aedes aegypti* (*Ae. aegypti*) increased susceptibility to the entomopathogenic fungus *Beauveria bassiana* (*B. bassiana* ) [180]. On the other hand, ectopic expression of *AaRel1-A*, one of two forms of Rel1 in *Ae. aegypti*, specifically activated Drosomycin resulting in increased resistance against *B. bassiana* [186]. *Toll5A* and *Spz1C* were also shown to be required for *B. bassiana* defense, as their knockdown increased the susceptibility to the fungus, in a manner similar to that of Rel1 [187]. *Ae. aegypti* Toll pathway also plays a significant role in regulating resistance to dengue virus [188-191]. Interestingly, a model of Wolbachia-mediated resistance to Dengue virus (DENV) in *Ae. aegypti* that involves Toll was described whereby *Wolbachia* infection leads to the induction of oxidative stress and elevated reactive oxygen species (ROS) levels in the mosquito host that activates Toll which is essential for the expression of antioxidants to counterbalance the resulting oxidative stress. This immune pathway also drives synthesis of AMPs that are involved in inhibition of *Dengue* virus proliferation in these *Wolbachia*-infected mosquitoes [192]. Toll and JAK/STAT pathways were also shown to be implicated in suppressing Zika virus infection [193]. Recently, a role of lipid droplets (LD) in the immune response of *Ae. aegypti* against bacteria and *Dengue* virus was described. Interestingly, there seems to be a direct link between signaling pathways and LD biogenesis as

evident from the increased LD content in midguts of mosquitoes with constitutively active Toll and Imd signaling [194].

b. Imd pathway:

In *Drosophila*, signaling through the Imd pathway is mainly activated through binding of diaminopimelic acid-type peptidoglycan (DAP-type PGN) from Gram-negative bacteria to the PGRP-LC dimer [115, 195-197]. PGRP-LCx homodimers sense polymeric DAP-PGN while LCx-LCa heterodimers detect short PGN end fragments [198], with LCx and LCa being distinct splice isoforms of the *PGRP-LC* gene [120]. Other PGRP family members play either negative (PGRP-LF[199, 200], PGRP-LB[201], and PGRP-SC family [202, 203]) or positive (PGRP-LE [204-206] and PGRP-LA[207]) roles in the activation of the Imd pathway. The intracytoplasmic cascade starts with the recruitment of the death domain protein Imd [208, 209] that further associates with the mammalian homolog of fas-associated protein with death domain (FADD) and with the caspase-8 homolog death-related ced-3/Nedd2-like protein (DREDD) [210, 211]. The latter is ubiquitinated by the E3-ligase inhibitor of apoptosis 2 (Iap2) [212] which associates with E2-ubiquitin–conjugating enzymes UEV1a, Bendless (Ubc13), and Effete (Ubc5) [213]. Activated DREDD cleaves Imd creating a novel binding site for Iap2, which can then K63-ubiquitinate Imd [212, 214]. These events ultimately lead to the recruitment and activation of the TAK1-binding protein 2/TGF β -activated kinase 1 (Tab2/Tak1) complex responsible for the phosphorylation and activation of the *Drosophila* IκB kinase (IKK) complex [215-218]. The IKK complex then phosphorylates Relish at multiple sites [217], an event that is key for efficient recruitment of RNA polymerase II to the promoters of Relish target genes [219]. In addition to phosphorylation, Relish activation requires cleavage of the

inhibitory C-terminal part, an event likely carried out by DREDD [220]. All the above culminate in the translocation of the active N-terminal part (Rel-68) into the nucleus where it triggers transcription of target genes encoding AMPs [220, 221]. While Akirin is required for Imd pathway function at the level of Relish [222], Pirk [223, 224], Caspar [225], and defense repressor 1 (Dnrl) [226] all function as negative regulators of the pathway. The Imd pathway bifurcates into the c-Jun N-terminal kinase (JNK) pathway at the level of Tak1 and Tab2 [227-229], eventually causing nuclear translocation of Activator protein 1 (AP-1) and activation of transcription of stress genes [167].

Although comparative genomic analysis revealed that the key components of the Imd pathway are conserved between *A. gambiae* and *Drosophila*, *Anopheles* Imd pathway doesn't fully mimic that in *Drosophila*. For instance, while *Anopheles* possesses two functional isoforms of Relish 2 (Rel2), flies have only one [230] and there is no reliable ortholog of TAB2 in *A. gambiae* [231]. Moreover, in contrast to *Drosophila* Relish, which responds solely to Gram-negative bacteria, the *Anopheles* full-length (Rel2-F) and shorter (Rel2-S) REL protein isoforms were shown to be involved in defense against both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria, respectively. Given that *Dif* is absent from *A. gambiae* mosquitoes, it appears that these mosquitoes use a single yet alternatively spliced NF-κB gene (*Rel2*) to mediate immune reactions for which *Drosophila* employs two distinct genes (Relish and *Dif)*. In addition to anti-bacterial defense, Rel2 is also implicated in anti-*Plasmodium* immunity [230]. Microarray analysis revealed that several immunity genes are regulated by mosquito Rel2 including, the antimicrobial peptides Cecrpoin1 (CEC1), CEC3*, Gambicin* (GAM1)*,* LRIM1, APL1*,* several clip domain serine

proteases (CLIPs), and FBNs among others [68, 184, 230]. A study investigating the role of PGRPLC (receptor of the Imd pathway) in mosquito infections with bacteria and malaria parasites revealed that PGRPLC is required for resistance to bacterial infections (*E. coli* and *S. aureus*). Interestingly, it was shown that signaling through PGRPLC regulates AMP expression at early stages of *S. aureus* but not *E. coli* infections. The proliferation of mosquito gut symbiotic bacteria population as well as intestinal bacterial infections were shown to be also under the control PGRPLC signaling. Moreover, silencing *PGRPLC* increased infection by malaria parasites indicating a role of PGRPLC signaling in modulating the intensity of mosquito infections with human and rodent malaria parasites [124]. Indeed, the Imd/Rel2 pathway seems to be the most effective against-*P. falciparum*. It has been shown that an over-activation of the pathway through either silencing *Caspar*, a negative regulator of the Imd pathway, confers a resistant phenotype in laboratory reared *A. gambiae*, *A. stephensi*, and *Anopheles albimanus* (*A. albimanus*) against *P. falciparum* [184]. In a study by Dong *et al.*, transgenic *A. stephensi* mosquitoes with blood meal-inducible expression of Rel2 were almost completely resistant to *P. falciparum*. This anti-*P. falciparum* activity is possibly due to the fact that Rel2 regulates the expression of key anti-*Plasmodium* factors [68]. Using RNAi, Garver *et al.* revealed that Imd, FADD, CASPL1, and Rel2 are the most effective players in the mosquito defense against *P. falciparum.* The study also demonstrated that the Imd pathway has the most potent activity against the parasite's ookinete stage, a reasonable activity against early oocysts, and a lesser activity against late oocysts. It was further established that the efficiency of the anti-parasitic responses obtained by silencing Imd pathway components and downstream effectors is dependent on infection intensity [231].

In *Ae. aegypti,* a tripartite relationship between the microbiota, immune system, and virus exists that impacts directly the outcome of the infection. Viral infections upregulate Rel2 in a microbiota-dependent fashion and constitutive activation of the Imd pathway decreases microbiota levels and increases Sindbis virus loads [232].

c. JAK-STAT pathway:

The Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway is one that has been conserved throughout evolution. In fact, the *Drosophila* JAK/STAT pathway has the same core components as in mammals, yet with less redundancy [233]. In the fly, the JAK-STAT pathway is initiated by binding of the extracellular ligands Unpaired (Upd) [234], Upd2 [235, 236] and Upd3 [237, 238]) to the predimerized transmembrane receptor Domeless (Dome) [239-241]. These molecules (Upd, Upd2 and Upd3) are induced locally upon tissue damage, in adult hemocytes upon bacterial challenge (Upd3), and in response to viral infections (Upd2 and 3) [237, 238, 242, 243]. Binding causes the receptor associated JAK tyrosine kinase Hopscotch (Hop) [244] to phosphorylate both itself and the cytoplasmic tail of the Dome receptor creating docking sites for the latent STAT92E proteins [245, 246]. Once bound to the receptor/JAK complex, STAT92E is in turn phosphorylated [241], dimerizes and translocates into the nucleus where it is capable of transcriptionally regulating the expression of target genes [246]. The *Drosophila* genome encodes three members of the suppressor of cytokine signaling (SOCS) protein family: Socs16D, Socs44A and Socs36E [247, 248], the latter being the main JAK-STAT negative feedback loop regulator [249, 250]. Another negative regulator that functions *via* a negative feedback loop is the protein tyrosine phosphatase PTP61F, a transcriptional target of the JAK/STAT pathway [251, 252]. The location and DNA-binding ability of

Stat92E are also subject to regulation. For instance, the *Drosophila* homologues of Ras-like guanine nucleotide-binding protein 3 (RanBP3) and RanBP10 control the signal-dependent nuclear transport of Stat92E [251]. In addition, Not4, homologue of the human C-C Motif Chemokine Receptor 4 (CCR4)- negative on TATA-less (NOT) transcription regulation complex subunit 4 (CNOT4), is needed for proper Stat92E DNA binding [253]. Activated Stat92E is also negatively regulated by PIAS, a homologue of mammalian protein inhibitors of activated STATs (PIAS) which are known to bind phosphorylated STAT dimers thereby blocking their binding to DNA [254, 255]. Moreover, sumoylation of *Drosophila* STAT92E has a repressive role in the regulation of the JAK/STAT pathway [256]. In *Drosophila*, a subset of Stat92E target gene promoters, containing Ken & barbie (Ken) binding sites that overlap with the sites of Stat92E, are subject to downregulation by Ken [251, 257]. Ken, an ortholog of the mammalian proto-oncogene B-cell lymphoma 6 (BCL6) can act as a repressor of STAT6-dependent target gene expression [258]. Other potential negative regulators of the JAK/STAT pathway are the *Drosophila* homologue of the bromo-domain-containing protein (BRWD3), Diedel, and PTP61F the homologue of human phosphotyrosine phosphatase B1 (PTPB1) [252, 259, 260].

A member of the STAT family that translocates to the nucleus and binds DNA in response to bacterial challenge was characterized in *A. gambiae* and annotated STAT1 [261]. A second *STAT* gene, annotated as *STAT2*, was revealed upon completion of the *A. gambiae* genome [123]. In a subsequent study examining the contribution of the STAT pathway in the mosquito immune response to bacteria and *Plasmodium,* STAT1, also known as AgSTAT-B, was shown to regulate the basal messanger RNA (mRNA) levels of *STAT2*, also known as AgSTAT-A. The study also demonstrated that

AgSTAT-A mediates the induction of nitric oxide synthase (*NOS*) and *SOCS* mRNAs in mosquitoes challenged with a mixture of *E. coli* and *Micrococcus luteus* (*M. luteus*), however, it is not required for mosquito tolerance to systemic or oral bacterial infections. On the other hand, silencing AgSTAT-A significantly enhanced infections with both *P. berghei* and *P. falciparum* parasites. Interestingly, the authors revealed that STAT signaling through AgSTAT-A is required for early parasite survival, but at the same time mediates lysis of early oocysts that complete development, thus limiting *Plasmodium* infection. An important effector in this STAT-regulated late phase anti-*Plasmodium* immunity was shown to be NOS [262].

The JAK-STAT pathway seems to modulate the defense of *Ae. aegypti* against *Dengue* virus infection. Suppression of the pathway through RNAi-mediated gene silencing of Dome and Hop renders mosquitoes more susceptible to the virus, whereas depletion of its negative regulator PIAS confers resistance to mosquitoes against viral infections. This anti-dengue activity is probably exerted through two JAK-STAT regulated and infection-responsive dengue virus restriction factors (DVRFs) [263]. In support of this study, transgenic mosquitoes genetically engineered to activate the JAK-STAT pathway by overexpressing either the receptor Dome or the Janus kinase Hop also showed an increased resistance to DENV infection [264].

d. JNK pathway:

The JNK pathway is a mitogen-activated protein kinase (MAPK) pathway highly conserved from mammals to insects. Several orthologs of components of this pathway were identified in *Drosophila* and *A. gambiae* [265, 266]. At the core of this signaling cascade is JNK, a MAP kinase that is activated by a MAPK kinase [265, 267-271]. JNK phosphorylates the transcription factors Jun and Fos, yielding a Jun/Fos dimer (AP-1

complex) that activates transcription of target genes [272]. A phosphatase, Puckered (puc), suppresses JNK signaling by dephosphorylating JNK. In fact, transcription of *puc* is regulated by JNK, thus making puc part of a negative feedback loop [270, 273, 274]. Among many different stimuli known to activate the JNK pathway, LPS was shown to be a key elicitor of JNK signaling in both *Drosophila* [228, 267-269, 271, 275] and *A. gambiae* [265]. In *A. gambiae* mosquitoes, a significant increase in the midgut expression of members of the JNK pathway, including *jnk*, *puc*, *jun* and *fos* was observed between 12–48 hours post infection with *P. berghei*. A modest change in Hemipterous (*hep*) expression was also detected. Moreover, pathway suppression through silencing either *hep*, *JNK*, *jun* or *fos* increased the prevalence of infection as opposed to silencing the suppressor *puc* that had the opposite effect. The study also demonstrated two key mechanisms by which the JNK pathway limits *Plasmodium* infection: First, it induces expression of the two enzymes [276] known to mediate midgut epithelial nitration in response to ookinete invasion, heme peroxidase 2 (HPX2) and NADPH oxidase 5 (NOX5) [277]. Second, it regulates expression of TEP1 and FBN9 [276], key components of the complement-like system that are produced by hemocytes and secreted into the hemolymph where they bind to the surface of ookinetes and initiate killing, possibly by lysis [138, 278]. Interestingly, the refractory *A. gambiae* L3-5 melanizing strain exhibited constitutive overexpression of JNK pathway genes, and gene silencing experiments confirmed that this pathway is indeed a key determinant of L3-5 refractoriness to *Plasmodium* [276]. On the other hand, JNK may mediate a yet to be defined anti-parasitic response in *A. gambiae* by regulating the expression of the Oxidation Resistance 1 (*OXR1*) gene [279], which is known for its protective role against oxidative damage in yeast and human cells, and is present in all

eukaryote genomes sequenced so far including *Drosophila* and *A. gambiae* [280]. In the latter, it was shown that the expression of OXR1 is induced upon oxidative stress, and that OXR1 along with JNK regulates expression of enzymes that detoxify ROS, with JNK acting upstream of OXR1. Moreover, *OXR1* silencing decreased *P. berghei* infection [279] in support of an earlier study [281]. This outcome is not surprising because OXR1 silencing would reduce catalase expression, hence increasing systemic levels of hydrogen peroxide ($H_2O_2$) and limiting infection. Unexpectedly, JNK silencing had the opposite effect, whereby it enhanced *Plasmodium* infection in the mosquito [279].

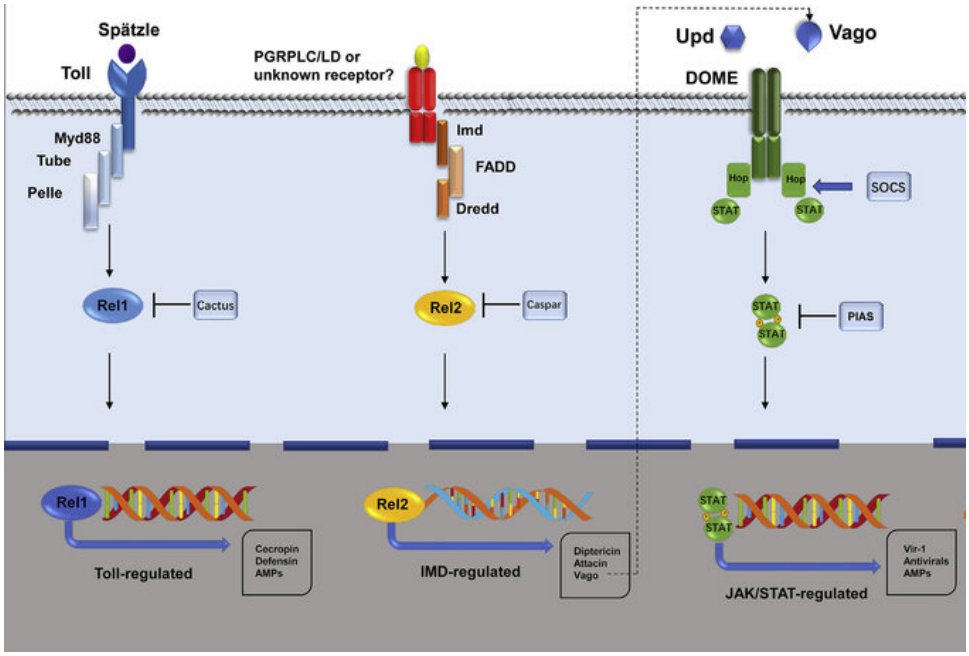


Figure 4. The main mosquito signaling pathways.

The Toll, Imd and JAK/STAT pathways constitute the major signaling transduction pathways in mosquitos. Signaling downstream of these pathways ultimately leads to the nuclear translocation of transcription factors, triggering activation of pathway specific genes that neutralize the invading pathogens or deal with tissue repair. (Adapted from [282])

### *3. Immune effector responses*

a. The complement-like response

Thioester-containing proteins (TEPs) are large secreted glycoproteins of the complement system with a characteristic intrachain β-cysteinyl-γ-glutamyl thioester bond [283] originally described in the human alpha-2-macroglobulin (α-2Ms), a protease inhibitor, and complement factor (C3), a central component of the complement system [284]. A number of factors including proteolytic cleavage render this bond reactive, allowing it to bind closely accessible hydroxyl or amine groups present at the surface of many biological entities including pathogens [285]. In addition to the complement factors and A2M subfamilies [285], the insect thioester-containing protein (iTEP) [286] and the macroglobulin complement-related (Mcr) [287], constitute a third subfamily inside the TEP superfamily [285]. In invertebrates, TEPs have been extensively studied in *A. gambiae* and *D. melanogaster* [288, 289]. Indeed, mosquito TEPs share structural and functional similarities with α-2Ms [283, 290]. The *A. gambiae* genome encodes 19 TEP gene homologs (*AgTep* 1–19), of which four pairs represent polymorphic variations rather than distinct genes (*AgTep1–AgTep16, AgTep5–AgTep17, AgTep6–AgTep18, and AgTep7–AgTep19*) [123, 291]. Perhaps the best functionally characterized of these is the highly polymorphic gene *TEP1* [289].

TEP1 is structurally similar to C3 and is a constitutively secreted hemolymph protein with a size of 165 kDa (TEP1-full) [116, 292]. Cleavage of the full form yields an 80 kDa active fragment (TEP1-cut) [116] that circulates in the hemolymph in association with two leucine-rich repeat (LRRs) proteins, LRIM1 and *Anopheles-Plasmodium*-responsive leucine-rich repeat 1 (APL1C) which form an obligate

disulfide-linked heterodimer [293-297]. *TEP1* knockdown (kd) in *A. gambiae* results in a 3- to 5-fold increase in *P. berghei* oocyst numbers in both susceptible and resistant mosquitoes [278, 298]. Interestingly, the knockdown phenotypes of all three genes *TEP1*, *LRIM1,* and *APL1* in one study was shown to be very similar: a 3-fold increase in *P. berghei* numbers in the mosquito midgut. Moreover, the coordinated action of LRIM1 and APL1 was shown to be crucial for binding of TEP1 to the surface of parasites and their subsequent lysis. Silencing either gene not only abolished TEP1 binding to and lysis of ookinetes but also led to depletion of the active TEP1-cut from the circulation and its deposition on self-tissues [293]. It is worth mentioning that the cleavage of TEP1 is independent of LRIM1 and APL1 function. It was recently reported that distinct members of the *APL1* gene family are utilized against different classes of *Plasmodium* parasites, with APL1A controlling the prevalence of *A. gambiae* infection with *P. falciparum* but not *P. berghei*, as opposed to APL1C that is required for protection against *P.berghei* and not *P. falciparum* [299]. TEP1 activity is positively and negatively regulated by the clip-domain serine protease homologs SPCLIP1 [300] and CLIPA2 [301], respectively. While CLIPA2 silencing trigger a TEP1-dependent melanotic response against *P. berghei* ookinetes exacerbating parasite killing in the midgut [301], silencing of *SPCLIP1* completely blocks ookinete melanization and enhances parasite development in the vector [300]. Moreover, TEP1 and SPCLIP1 localization on dead parasites is mutually dependent [300]. SPCLIP1 and CLIPA2 seem to function as positive and negative regulators of a yet unidentified TEP1 convertase that catalyzes TEP1-F cleavage to the active $TEP1_{cut}$ form [300]. It has been suggested that TEP1-cut acts as part of a convertase that catalyzes the activation of other

TEP1 molecules in the proximity of pathogens and that the LRIM1/APL1C heterodimer regulates formation of this TEP1 convertase [296].

The JNK pathway was shown to control the basal expression levels of TEP1 in hemocytes thus exerting tight control over TEP1 at the transcriptional level [276]. In addition, it was shown that Rel1 and Rel2 are required for the constitutive expression of *TEP1* prior to parasite invasion of the midgut epithelium [183]. Interestingly, a novel functional link between lipid carrier proteins, complement and JNK signaling has been highlighted, whereby silencing of Apolipoprotien II/I (*Apo-II/I*) increased the expression of *TEP1* following systemic infections with *E. coli* and *B. bassiana* in a Jun dependent manner [302].

*TEP1* is a highly polymorphic gene in the *A. gambiae* field population, with multiple alleles conferring variable degrees of mosquito resistance to malaria parasites. Two major alleles, *TEP1s* and *TEP1r*, were originally identified in laboratory mosquito strains based on being either susceptible (G3) or refractory (L3-5) to *P. berghei* infection [278]. Indeed, a subsequent study identified different TEP1 alleles in different laboratory strains: *TEP1*S1* (PEST) , *TEP1*S2* (4Arr) , *TEP1*S3* (G3) , *TEP1*R1* (L3–5) , and *TEP1*R2* (4Arr) [298]. Several studies pinpointed to a role of *TEP1*R1* in conferring the highest levels of resistance among all alleles [298, 303-305]. Despite the key role of TEP1 in *Plasmodium* killing, transgenic mosquitoes overexpressing *TEP1* did not exhibit increased resistance to *Plasmodium* [306], suggesting that the amount of TEP1 available in the hemolymph to attack *Plasmodium* ookinetes is not limited.

How TEP1 recognizes microbial surfaces including ookinetes remains unknown. However, epithelial nitration of invading ookinetes by HPX2 and NOX5 is thought to

modify ookinete surfaces making them “visible” to TEP1 [277]. In fact, it was shown that, epithelial nitration and TEP1-mediated lysis (Fig. 5), which are often thought to be mutually exclusive [307] rather work sequentially. Silencing either HPX2 or NOX5 abolished TEP1-mediated lysis. In addition, HPX-2 silencing significantly reduced the proportion of parasites labeled with TEP1 as well as *Plasmodium*-induced TEP1 binding to the mosquito midgut [277]. JNK signaling induces the expression of both HPX2 and NOX5 in mosquito midgut cells [276], further corroborating the role of this pathway in anti-*Plasmodium* immunity. Several lines of evidence implicate TEP1 in parasite killing. TEP1 binds ookinetes after they traverse the midgut epithelium; this binding is temporally correlated with the appearance of morphologically degenerate ookinetes and is associated with two respective peaks of transcriptional upregulation. In addition, the vast majority of TEP1-bound ookinetes do not express the vital marker, green fluorescent protein (GFP), and most importantly, *TEP1* kd increases parasite numbers and completely abolishes their melanization [278]. Nevertheless, additional TEP1-independent parasite killing mechanisms do exist, as immunofluorescence assays in dissected midguts from wildtype mosquitoes have detected dead parasites that are not bound by TEP1 [289].

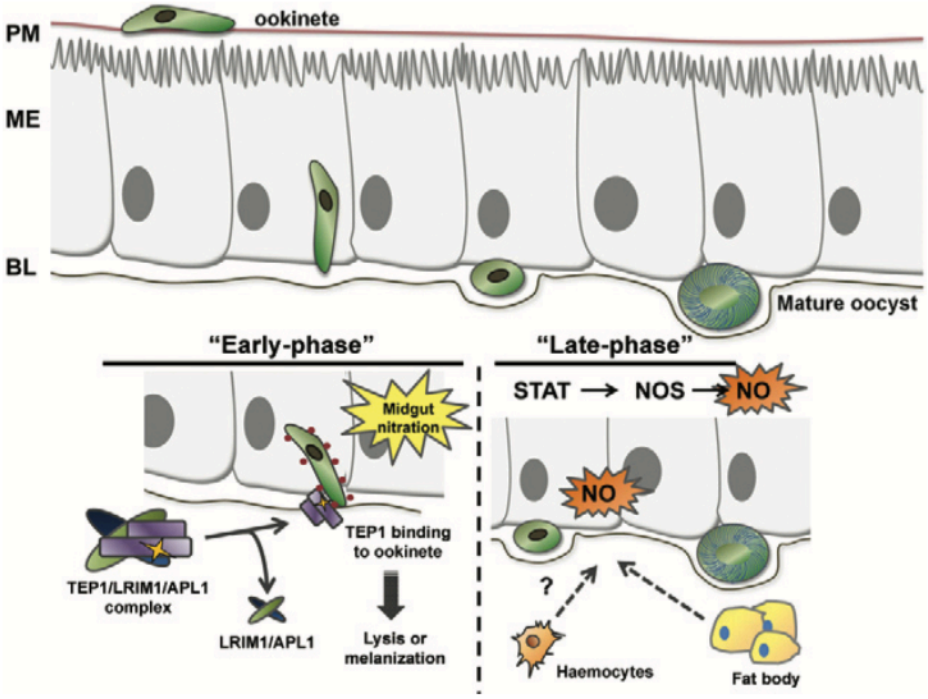


Figure 5. Mechanisms of "early-phase" and "late-phase" immunity.

After traversal of the midgut epithelium, parasites are subjected to two "phases" of the mosquito innate immune response. limits ookinete survival is limited by an "early- phase" immunity before or at the transition to oocysts. As ookinetes traverse the midgut epithelium, they undergo nitration (red dots) and are "marked" for immune recognition by complement-like proteins circulating in the mosquito hemolymph including TEP1. Following recognition, TEP1 binds to the ookinete initiating lysis or melanization. A "late-phase" immune response involves the synthesis of nitric oxide (NO) by the STAT pathway and limits oocyst survival. It is unclear to what extent do each of the midgut, fat body and hemocytes contribute to this "late-phase" response. (Adapted from [17])

*P. falciparum* strains differ in their ability to infect *A. gambiae* mosquitoes. For instance, the African NF54 strain was shown to be resistant to TEP1 mediated killing in refractory L3-5 mosquitoes as opposed to the Brazilian 7 G8 strain that demonstrated high susceptibility to TEP1 [139, 305], suggesting that some means to evade TEP1

killing have evolved in African parasites which are sympatric with *A. gambiae* [305]. Moreover, another study based on the laboratory NF54 strain revealed that there is a correlation between resistance of the parasites to TEP1 and a polymorphism in *P. falciparum* 47 (*Pfs47*) gene. In addition, it was shown that Pfs47 is necessary for *P. falciparum* parasites to evade two well-characterized TEP1-mediated immune responses: killing followed by melanization in the refractory L3-5 strain and parasite lysis without melanization in the susceptible strains [308]. A more recent study examining resistance of *P. falciparum* isolates of African origin (NF54, NF165 and NF166) to TEP1-mediated killing in susceptible (S) and refractory (R) *A. gambiae* strains revealed that all parasite clones developed successfully in S mosquitoes with limited impact of TEP1 on transmission efficiency. On the other hand, a reduction in oocyst numbers of NF166 and NF165 was observed in R mosquitoes that can be reverted by silencing *TEP1*. These results clearly show a difference in the capacity of different African *P. falciparum* strains to evade TEP1-mediated killing in R mosquitoes. However, no significant correlation between *Pfs47* genotype and this resistance was established [309].

Not only is TEP1 a hallmark of mosquito immune effector responses to both rodent and human malaria parasites, but also to systemic bacterial infections [116, 139, 310]. In fact, *TEP1* kd mosquitoes are less tolerant and resistant to systemic *E. coli* infections. In addition to binding to the surface of *E. coli* bioparticles, TEP1 also regulates the recruitment of its regulators CLIPA2 and SPCLIP1 to these surfaces [301]. TEP1 recruitment to bacterial surfaces was shown to be associated with bacterial lysis [302]. It has been also shown that TEP1 promotes the phagocytosis of Gram-negative and Gram-positive bacteria by cultured mosquito cells, and by hemocytes *in vivo*. For

instance, phagocytosis of *E. coli* is reduced by 2-fold after incubation of mosquito cells with conditioned medium (CM) specifically depleted of TEP1 [116]. Likewise, this cellular response is dramatically impaired in *TEP1* kd mosquitoes. In fact, it was shown that *TEP1* kd reduced the efficiency of phagocytosis of *E. coli* and *S. aureus* by ~60% and ~40%, respectively [310]. In this respect, TEP1 seems to play a very similar role to C3, binding to bacterial surfaces and promoting their lysis and phagocytosis [289].

TEP1 has also demonstrated a role in anti-fungal immunity whereby silencing *TEP1* and *CLIPA8* was shown to completely abolish melanization of fungal hyphae and increase mosquito susceptibility to natural *B. bassiana* infections. Interestingly, microscopic analysis revealed specific association of TEP1 with hyphal surfaces and its requirement for phenoloxidase recruitment to these surfaces [311].

b. Antimicrobial peptides

A hallmark of insect humoral immunity is AMP synthesis. These are either produced locally by barrier epithelia or systematically in the fat body from where they are secreted into the hemolymph. In general, there are two modes of action through which insect AMPs exert their antimicrobial effects: Peptide-lipid interaction or receptor-mediated recognition processes [312]. The *A. gambiae* genome encodes four cecropins (Cecs), four defensins (Defs), one attacin and one GAM [123].

Bacterial challenge with Gram positive *M. luteus* induces the expression of *Cec* genes as evident from elevated RNA levels (4-fold as compared to controls) observed 2 hrs post-infection of *A. gambiae*. Moreover, cecropin expression was also elevated 24 hrs post infection with *P. berghei*. Peptides corresponding to mature *A. gambiae* cecropin displayed activity against a panel of microorganisms including Gram-negative and Gram-positive bacteria, filamentous fungi and yeasts [313]. Kim *et al.*

reported a 60% reduction in the number of *P. berghei* oocysts in transgenic *A. gambiae* mosquitoes manipulated to express CecA 24 h after a blood meal in the posterior midgut [314], suggesting that the manipulation of endogenous mosquito genes (in this case an antimicrobial peptide) may be a valuable tool to engineer strains that are refractory to malaria infection. Gwadz *et al.* (1989) also reported a reduction in the number of *Plasmodium* sporozoites in *A. gambiae* mosquitoes injected with CecB [315].

A recombinant *A. gambiae* Def peptide demonstrated antimicrobial activity against Gram-positive but not Gram-negative bacteria (Apart from some *E. coli* strains) nor yeast. It also exhibited a strong bactericidal effect on *M. luteus* and fungicidal effect on *Neurospora crassa* (*N. crassa*) spores [316]. Induction of defensin was observed in the abdomens and salivary glands of *A. gambiae* at 15 and 20 days post *P. berghei* infection, when sporozoites are released from the midgut into the hemolymph and invade the glands [317]. Also in *A. gambiae*, substantial increase in defensin mRNA levels was observed 20–30 h both locally and systematically after ingestion of a *P. berghei* infected blood-meal, presumably in response to parasite invasion of the midgut epithelium [318, 319]. It was also demonstrated that Defensin expression is induced in response to bacterial infection [319, 320]. Reverse genetics confirmed an anti-bacterial role for Defensin against Gram-positive but not Gram-negative bacteria, however, it didn't seem to be a major antiparasitic factor in *A. gambiae in vivo* [321].

Gambicin demonstrated significant antimicrobial activity against Gram-negative *E. coli* and Gram-positive *M. luteus*, and a morphogenic effect against a filamentous fungus. It also enhanced the lethality of *P. berghei* ookinetes (2-fold relative to the control). Transcription of this gene was significantly up-regulated upon infection with *P. berghei* in the midgut at 24 h after an infectious blood meal which coincides with

ookinetes invasion. Similarly, induction was also observed in cell lines challenged with heat-killed *E. coli*, *M. luteus*, LPS, or lipoteichoic acid (LTA) [182]. Arrighi *et al.* found that following ingestion, *P. falciparum* glycosylphosphatidylinositol (GPI) induces a strong expression of several *A. gambiae* antimicrobial peptides including *Def1, CecA*, and Gambicin. LPS also up-regulates the expression of *CecA*, and *Gambicin* but not that of *Def1* [322].

c. Reactive oxygen and nitrogen species

Two very important effectors of the mosquito immune response are RNS and ROS. RNS includes nitric oxide, a free radical produced during the oxidation of L-arginine to L-citrulline by an enzyme called NOS [323]. In *A. stephensi* mosquitoes, *NOS* is a single copy gene that is alternatively spliced into 18–22 distinct transcripts [324]. Local and systemic transcriptional activation of *NOS* has been reported in strains of *A. stephensi* susceptible to *P. berghei* infection [325]. Early induction is likely the result of midgut bacterial growth coincident with blood feeding given that infection significantly enhanced AsNOS expression in the midgut at 1, 2 and 3 days post blood meal (pBM); only the increase at 1 day pBM was not statistically significant. Increases in AsNOS expression also occurred at later time points (day 9) that correspond to the initiation of sporozoite release form mature oocysts. Moreover, circulating levels of end-products of nitric oxide (NO) synthesis (nitrite/nitrate) were elevated in mosquitoes infected with *P. berghei*. Dietary provision of a NOS inhibitor to *P. falciparum*-infected females significantly increased the number of developing oocysts, confirming that NO limits *Plasmodium* development in *A. stephensi*. Diaphorase staining revealed AsNOS activity in individual cells in the posterior midgut (the region where *Plasmodium* development typically occurs) at 24 h after feeding on either *P. berghei* or *P. falciparum* [325]. Two

subsequent studies revealed that induction of AsNOS expression in the midgut begins as early as 6 hrs post-infection (p.i.) with *P. berghei* [326, 327]. Later on it was demonstrated that following ookinete invasion, several cells protrude to the luminal side of the midgut, exhibit very high levels of NOS, and are closely associated with ookinetes positive for the GPI-anchored surface protein of *Plasmodium berghei* (Pbs21). Moreover, these cells exhibit abnormal nuclear morphology, fragmented DNA and radically reorganized actin cytoskeleton as part of a series of toxic reactions leading ultimately to cell death [328]. A subsequent study in *A. stephensi* revealed that some of the ookinete-invaded cells (protruding and expressing high NOS levels) undergo protein nitration [329], a process mediated by a peroxidase activity in the presence of nitrite and H2O2. Interestingly, a time lag seems to exist between NOS expression and protein nitration; when a parasite migrates laterally and invades more than one cell, tyrosine nitration was often confined to the first cell invaded by the parasite despite the fact that all other cells have already protruded and upregulated NOS expression. Similarly, the pattern of induced peroxidase activity coincides with that of tyrosine nitration; the peroxidase activity is usually much higher in the cell that was invaded first [329]. Ookinete invasion of the midgut elicited similar responses in *A. gambiae* characterized by cell protrusion, localized peroxidase activity, and tyrosine nitration [329]. Ookinete-induced peroxidase activity correlated with the transcriptional activation of several peroxidase genes. The authors suggested that ookinete-invaded cells undergo protein nitration, catalyzed as a two-step reaction. Ookinete invasion induces NOS expression, which catalyzes NO production. NO is very unstable and converts readily to nitrite which, along with hydrogen peroxide, serves as substrates for inducible peroxidases that mediate tyrosine nitration. Nitrated cells

undergo nuclear degeneration and eventually bud off into the midgut lumen. In susceptible mosquito strains, the delay between NOS and peroxidase induction most probably offers a limited time window for ookinetes to exit invaded cells before tyrosine nitration takes place [329]. Recently, HPX2 and NOX5 were identified as key enzymes that together with NOS mediate protein nitration in ookinete-invaded midgut cells of *A. gambiae* (G3) [277]. It was previously shown that disruption of the immunomodulatory peroxidase (IMPer)/ Dual oxidase (Duox) system leads to a dramatic induction of NOS and reduction in *P. falciparum* infection [330]. Co-silencing *IMPer/HPX2* or *IMPer/NOX5* decreased nitration to control levels further emphasizing that the HPX2/NOX5 system is indispensable for efficient nitration and NOS induction alone is not sufficient to achieve an effective anti-*Plasmodium* response [277]. NOS is immune responsive not only to *P. berghei* infections but also to infection with a mixture of *E. coli* and *M. luteus* [317]. Similar upregulation of *NOS* in response to systemic infection with *E. coli* and *M. luteus* was reported in a more recent study [331]. NOS is abundant in the granulocyte subpopulation of hemocytes in *A. gambiae*, and bacterial challenges were shown to increase the percentage of NOS-stained cells as well as staining intensity. Inhibition of nitric oxide synthesis decreased mosquito tolerance and resistance to *E. coli* infections indicating that NOS is essential for defense against systemic bacterial infections [331]. It is noteworthy to mention that the transcriptional activation of NOS in *A. gambiae* is regulated by STAT-A in response to bacterial and *Plasmodium* infections. NOS is also an important effector of a STAT-regulated late-phase immune response against *Plasmodium* oocysts in *A. gambiae* (Fig. 5). In fact, a minimal level of NOS activity was required for survival of early stages of *P. berghei*, but once oocysts form, reducing NOS expression enhanced parasite survival and

rescued the effect of silencing the STAT suppressor SOCS. Moreover, NOS was found to be homogeneously expressed in the cytoplasm of midgut epithelial cells as well as in the carcass but not in hemocytes [262]. Interestingly, it has been shown that both *AsNOS* expression in *A. stephensi* cells and parasite development in this mosquito are regulated by mammalian latent transforming growth factor (TGF-β1) ingested during the process of blood-feeding [327]. In a subsequent study, the authors demonstrated blood feeding and the accumulation of oxyhemoglobin trigger the production of nitric oxide in the mosquito midgut which limits parasite development [332]. In addition to human-derived factors, it has been shown that *P. falciparum* GPIs (PfGPIs) can induce *AsNOS* expression *in vitro* and *in vivo* by activating kinases associated with insulin signaling [333]. Another prominent parasite-derived signal in *Anopheles* is Hemozoin (Hz) that was also shown to induce AsNOS gene expression in immortalized *A. stephensi* and *A. gambiae* cell lines *in vitro* and in *A. stephensi* midgut tissue *in vivo*. The authors also reported that signaling pathways activated by PfGPIs and Hz have both unique and shared components [334].

NADPH oxidase/Nox or Duox family of proteins constitute two classical sources of ROS associated with the immune response in *A. gambiae* [277, 330]. Another significant source of ROS are mitochondria, the main cellular energy-transducing site. In fact, silencing the mitochondrial carrier 1 (AgMC1), a member of the solute carrier family 25 (SLC25) family in *A. gambiae*, altered mitochondrial coupling and elicited metabolic changes that decreased ROS production in the mosquito midgut promoting the survival of *P. berghei* [335]. A member of the microbial flora (*Enterobacter*) in wild *A. gambiae* mosquito populations was shown to interfere with *Plasmodium* development through ROS production, revealing a mosquito-independent process of

ROS generation that increases mosquito resistance to malaria parasites [336]. Furthermore, Kumar *et al.* reported that a key physiological difference between *A. gambiae* S and R strains could be ROS detoxification. The different strains exhibited morphological differences including fewer and smaller peroxisomes in pericardial cells of R mosquitoes. Moreover, the authors demonstrated that the melanotically encapsulating R strain is under a chronic state of oxidative stress, which is exacerbated by blood feeding. Direct measurements of ROS in the hemolymph confirmed that the R strain harbored significantly higher basal levels of hydrogen peroxide than the two susceptible strains S and G3, and that induced H2O2 levels were highest in the R strain at 24 h after a blood meal. In addition, dietary administration of an antioxidant, vitamin C, not only decreased H2O2 levels but also the melanotic encapsulation of sephadex beads and *Plasmodium* parasites. Moreover, there is a correlation between midgut mRNA induction of catalase and super oxide dismutase 2 (CuZnSOD2) in response to blood feeding and ROS levels in a given strain [337]. In addition to mediating anti-*Plasmodium* activities, ROS are also required to mount effective antibacterial responses, as reduction of ROS by dietary administration of antioxidants significantly decreased survival after a bacterial challenge [338]. Indeed, several ROS detoxification enzymes were induced after a blood meal and this induction was further increased upon infection with *P. berghei* except for catalase, a major enzyme involved in H2O2 detoxification, whose expression was suppressed in *P. berghei*-infected midguts. Further reduction of catalase expression by RNAi led to an increase in H2O2 levels and promoted parasite clearance through a lytic mechanism that does not involve melanization. In addition, the high mosquito mortality often observed after *P. berghei* infection in G3 mosquitoes

appears to result in part from excess production of ROS, and can be reduced by oral administration of uric acid [338].

d. Melanization

Melanization is an immune effector response that is triggered locally, for instance by an injury to the cuticle, or systemically following hemolymph invasion by microbes. It involves the rapid synthesis and deposition of a black-brown pigment (melanin) around invading pathogens or at the site of injury. In insects, there appears to be a crosstalk between the coagulation system and melanization whereby the former initiates the clotting process and the latter is required for strengthening and stabilizing the primary clot (reviewed in [339]). Besides its role in immunity, melanization has been shown to contribute also to cuticlar sclerotization [340].

i. Melanin biosynthesis pathways in insects

In insects, melanogenesis is initiated by the hydroxylation of phenylalanine to tyrosine, a reaction catalyzed by phenylalanine hydroxylase (PAH). Tyrosine is then hydroxylated by phenoloxidase (PO) to dihydroxyphenylalanine (Dopa) which is further oxidized to dopaquinone by the same enzyme. In the presence of thiol compounds, dopaquinone forms cysteinyl and glutathional conjugates that give rise to yellow-red pheomelanins. Otherwise, dopaquinone converts spontaneously to dopachrome, which is in turn decarboxylated by dopachrome conversion enzyme (DCE) into 5,6-dihyroxyindole (DHI).This is followed by oxidation of DHI to indolequinones that eventually undergo non-enzymatic polymerization to produce the brown–black heteropolymer, eumelanin. In an alternative pathway, Dopa formed by the hydroxylation of tyrosine is decarboxylated by Dopa decarboxylase (DDC) to form

dopamine, from which molecules that are involved in cuticular sclerotization are derived (reviewed in [341]) (Fig. 6). Melanization ultimately leads to pathogen death either by oxidative damage resulting from unstable melanogenic intermediates (reviewed in [342]) or by starvation following confinement of the pathogen in a melanin capsule, hence isolating it from the nutrient-rich surroundings [343].

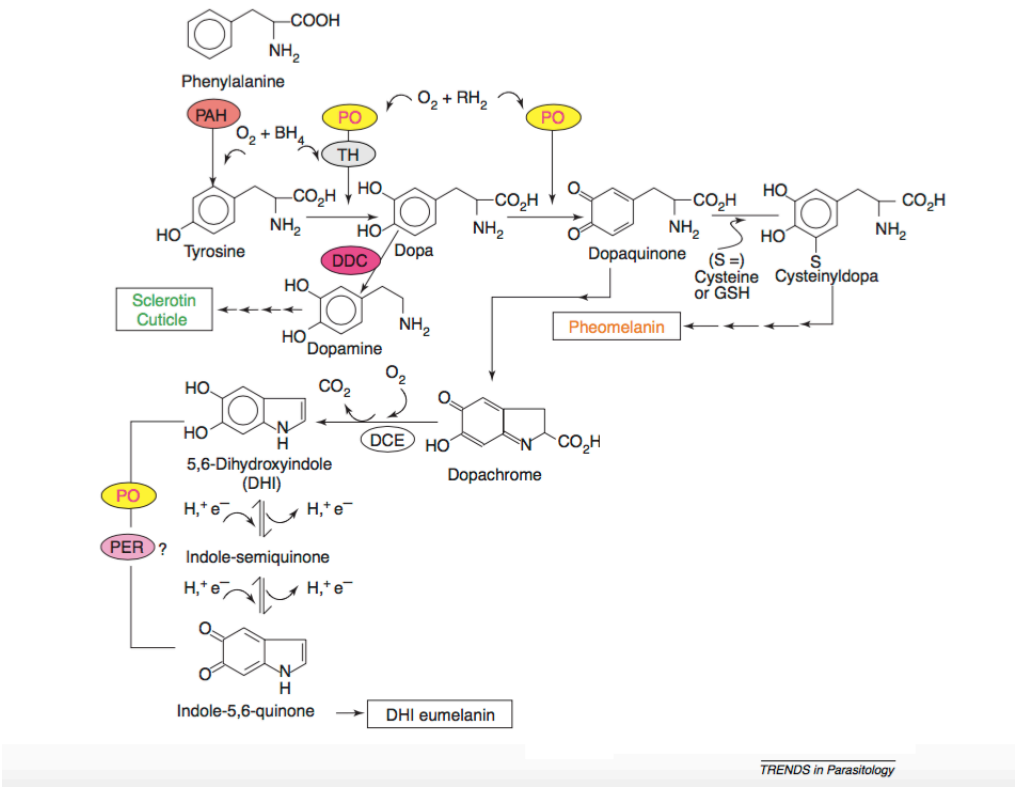


Figure 6. Generalized biochemical pathways of melanin biosynthesis.

In mosquitoes and other insects, melanin biosynthesis is initiated by hydroxylation of phenylalanine to tyrosine by PAH using tetrahydrobiopterin (BH4) and dioxygen as co-substrates. PO then converts tyrosine to dopa and dopaquinone. Tyrosine hydroxylase (TH) also mediates tyrosine conversion to dopa. Dopaquinone forms cysteinyl and glutathional conjugates that result in synthesis of yellow–red pheomelanins in the presence of thiol compounds. In the absence of these compounds, dopaquinone converts spontaneously to dopachrome, which then undergoes decarboxylation by DHI and indole-5,6-quinone to give rise to black eumelanin. In insects, the formation of DHI is accelerated by DCE. DHI

melanin in addition to molecules involved in cuticular sclerotization are derived from dopamine through the action of DDC. (Adapted from [341])

ii. Prophenoloxidase activation pathways in model insects

A key enzyme in melanin biosynthesis is phenoloxidase (PO) which catalyzes the hydroxylation of tyrosine to Dopa and the oxidation of Dopa to the respective quinones (reviewed in [341]). PO is synthesized as a prophenoloxidase (PPO) zymogen that is cleaved by a prophenoloxidase activating proteinase (PAP) into active PO (Fig. 7).

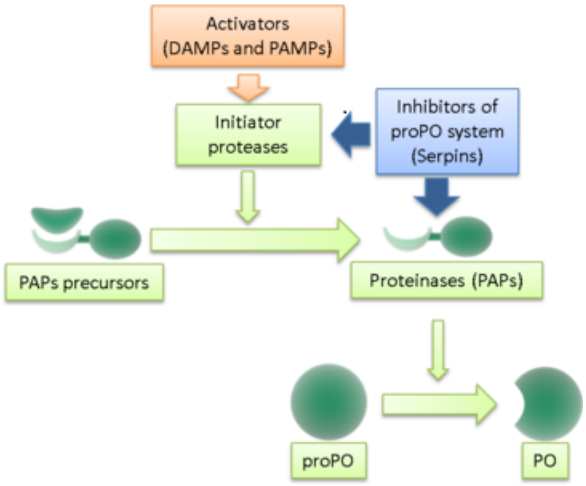


Figure 7. Schematic overview of the activation of PPO and melanogenesis in insects. PRRs recognize PAMPs leading to the activation of initiator proteases which in turn activate a protease cascade that results in conversion of inactive PAP zymogens to active proteinases. PPO is cleaved by active PAPs to form active PO. (Adapted from [344])

Prophenoloxidase activating proteinases contain one or two clip domains and thus are considered as members of the CLIP family. Members of this family share a common feature: All contain at least one disulfide-bridged structure that assumes the

shape of a paper clip, called the clip domain. The regulatory amino-terminal clip domain is connected by a linker sequence of variable length to a catalytic serine proteinase domain at the carboxyl terminus. CLIPs are synthesized as zymogens that must undergo specific proteolytic cleavage to become activated (reviewed in [345]) (Fig. 8). Catalytic CLIPs are referred to as cSPs (clip domain containing serine proteinases) whereas those that lack one or more of the three residues (His, Asp, Ser) that form the catalytic triad are considered non-catalytic and are called clip-domain containing serine proteinase homologs (cSPHs) (reviewed in [346]). CLIPs can be classified into five clades A–E, whereby CLIPs B, C, and D are catalytically active while CLIPs A and E are non-catalytic [300]. All characterized insect PPO activation cascades generally share a conserved pattern of hierarchical activation. The most upstream proteinase that is likely to interact with and relay information from a PRR is a non-CLIP ModSp [169, 347-349]. ModSps, which are generally autoactivated, activate a CLIPC, through proteolytic processing in the linker region, which in turn activates a CLIPB. The PAP is always a CLIPB (reviewed in [350]). Serine proteinase inhibitors known as serpins tightly regulate CLIP cascades that control PPO activation. In fact, serpins constitute the largest family of serine proteinase inhibitors in higher eukaryotes regulating several biological functions, including reproduction, developmental processes, hematophagy, cellular secretion, and immunity (reviewed in [351]). Below we provide a brief description of these cascades in model insects in which they have been best characterized.

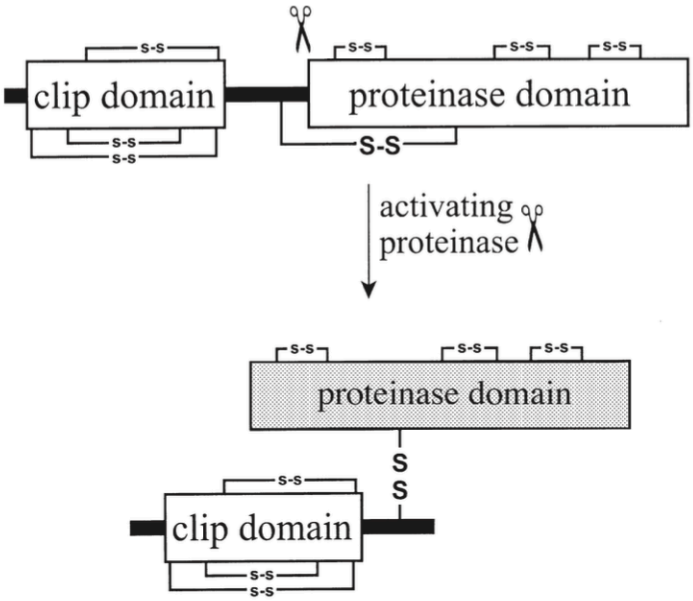


Figure 8. Domain organization of clip-domain proteinases.

CLIPs are composed of at least one amino terminal clip domain connected to a carboxyl-terminal serine proteinase domain by a linker region. A disulfide bond connects the linker region to the proteinase domain such that the clip domain and proteinase domain remain covalently attached when the zymogen is activated by specific proteolysis in the proteinase domain. (Adapted from [345])

- *Drosophila melanogaster*

Tang *et al.* characterized two immune inducible melanization proteases (MP1 and MP2) that define a melanization cascade regulated by the serpin Spn27A in *Drosophila*. The proteases are activated by microbial infection and function sequentially to activate melanization and PO activity in the fruit fly, with MP2 functioning genetically upstream of MP1. While MP2 seemed to be specifically involved in the anti-fungal response, MP1 was shown to be required in response to both bacterial and fungal infections [352]. In addition to Spn27A, Spn77Ba was also shown to regulate melanization locally in the epithelium of the fly respiratory tract through inhibiting a

protease cascade involving MP1 and MP2 [353]. However, the PRR as well as the ModSp acting upstream of this cascade remains unidentified [346]. Another CLIP serine protease, Hayan, was shown to be required for both wound- and microbe-induced PO activation in *Drosophila.* Interestingly, *Hayan* mutation was capable of suppressing the spontaneous melanization phenotypes observed in *Spn27A* as well as in *Spn77Ba*-mutant flies. In addition, it was demonstrated that melanization of *Spn28D* mutants could be completely abolished in a *Hayan* mutant genetic background [354]. A study investigating the role of SPs in the melanization response using compound mutants in *Drosophila* revealed that Hayan, acting through both PPO1 and PPO2, is necessary for the blackening reaction after clean injury. Upon septic injury with the Gram-positive bacterium *M. luteus,* residual melanization that requires both PPO1 and Sp7 can be observed, even in the absence of Hayan. A combined role of Hayan and Psh in propagating the signal downstream PRRs activating either Toll signaling or melanization was revealed as well, a clear indication of crosstalk between the two pathways (Fig. 9) [355].

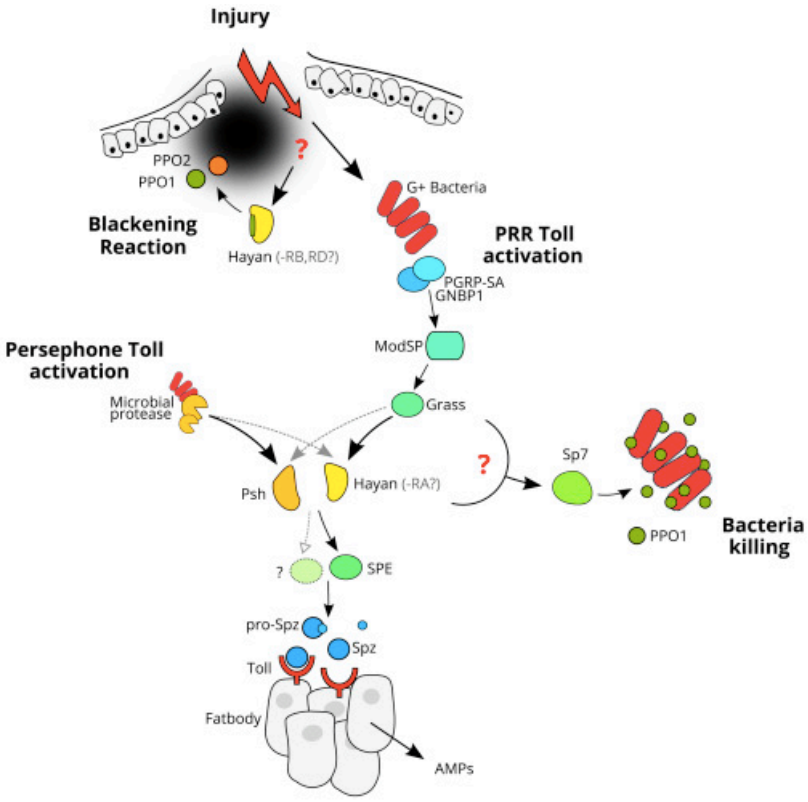


Figure 9. A revised model of SPs regulating the Toll pathway and the melanization reaction.

Upon wounding, Hayan can be activated by an unknown mechanism that results in the deposition of melanin around the wound area (top left), a reaction that can be achieved through both PPO1 and PPO2. If Gram-positive bacteria enter through the wound, peptidoglycan can be recognized by the PRRs PGRP-SA, and GNBP1, initiating the sequential activation of the SPs ModSP, Grass Psh/Hayan, and SPE ultimately leading to the activation of Toll signaling in the fat body (middle). This extracellular SP pathway likely branches at the position of Hayan and Psh to Sp7, activating PPO1 to combat invading bacteria (right). Microbial proteases can activate the Toll pathway through the Psh-SPE-Spz extracellular pathway. It is unclear whether microbial proteases can also activate Hayan. However, both Hayan and Psh regulate the Toll pathway downstream of Grass, ModSP, and PRRs. A previous study [356] and our data suggest the existence of another SP capable of cleaving Spz beyond SPE (Adapted from [355]).

- *Manduca sexta*

In *Manduca sexta* (*M. sexta*), two β-1,3-glucan recognition proteins (βGRP1 and βGRP2) were shown to be involved in PPO activation. Upon binding to soluble β1,3-glucan, a molecular pattern specific for fungi, βGRP1 serves as a pattern recognition molecule and ultimately triggers the activation of the PPO pathway [357]. It was reported as well that another protein, βGRP-2, binds laminarin, a β-1,3-glucan from fungi, and lipoteichoic acid to aggregate yeast and bacteria. In addition, Laminarin-triggered PPO activation was greatly enhanced in the presence of βGRP-2 [358]. Wang *et al.* later reported that interaction of β-1,3-Glucan with βGRP2 activates Hemolymph Proteinase 14 (HP14), an initiation enzyme of the PPO activation system in *M. sexta* [359]. This is in agreement with a previously proposed role for HP14 as a component of the PPO activation system in *M. sexta* [347]. HP14 is a ModSp which in turn cleaves the HP21 zymogen (proHP21) generating active HP21 which activates PPO activating proteinase-2 precursor (proPAP-2). The latter generates active PO in the presence of *M. sexta* clip domain serine proteinase homologs SPH-1 and SPH-2 [360]. HP21 was also identified as a candidate activator of proPAP3 [361]. It is worth mentioning here that, three PAPs (PAP-1, 2 and 3) have been shown to be direct activators of PPO in *M. sexta* [362-364]. PAP-1 activation is carried out by HP6, a *M. sexta* proteinase activated in response to microbial infection. HP6 also activates another proteinase, HP8, stimulating a Toll-like pathway that leads to the synthesis of AMPs [365]. In addition to the aforementioned role of SPH1 and SPH2 in PAP-2-induced PPO activation, other studies pointed to a similar function of these so-called cofactors in enhancing PPO cleavage by PAP-1 [366, 367] and PAP-3 [367]. Wang *et al.* proposed a positive feedback mechanism in which adding minute amounts of PAP-1 to larval plasma drastically enhances PPO activation. The enhanced PPO activation was accompanied with the

proteolytic activation of HP6, HP8, PAP1, SPH1, SPH2 and PO precursors. The authors proposed a model for PAP1-boosted PPO activation in which PAP1, together with an unknown protein, acts on HP6 zymogen proHP6 to generate HP6 which in turn cleaves and activates HP8 zymogen proHP8 and proPAP1 as well as SPH2 precursor (proSPH2) [368]. A subsequent study revealed a similar positive feedback loop for PAP-3, whereby PAP3 activates proPAP3 to generate more PAP3 which cleaves SPH1 precursor (proSPH1) and proSPH2 to form an SPH complex that acts as a cofactor for PPO activation by PAPs. Also, PAP3 indirectly activates proHP6, the active form of which then activates proPAP1 and proSPH2 as previously mentioned [369]. Another layer of regulation during PPO activation in *M. sexta* is carried out by serpins. PAP-3 has been found to be regulated by each of serpin 1J [364], serpin-3 [370], serpin-6 [371], and serpin-7 [372].Two additional serpins, serpin-4 and serpin-5, were shown to regulate the PO activation pathway by inhibiting one or more target proteases upstream of PAPs [373]. While all three proteases HP-1, HP-21, and HP-6 were identified as target proteases of serpin-4, serpin-5 was found to inhibit only HP-1 and HP-6 [374]. It is worth mentioning that HP1 zymogen proHP1 was shown to participate in the PPO activation system, probably through cutting proHP6 to generate HP6 that in turn activates proPAP1 and proHP8 [375] (Fig. 10).

- *Tenebrio molitor*

In *T. molitor*, DAP-PGN, Lysine peptidoglycan (Lys-PGN), and β-1,3-Glucan but not LPS were shown to activate the melanization cascade. In addition, the authors identified a *Tenebrio molitor* (Tm) -PGRP highly homologous to *Drosophila melanogaster* (Dm)-PGRP-SA that recognized both Lys- and DAP-PGN thus functioning as a common PGN recognition molecule of Lys- and DAP-PGN-dependent

melanization responses [376]. A subsequent study revealed that partial digestion of Lys-type PG of Gram-positive bacteria triggers the formation of PGRP-SA clusters on the bacterial surface that ultimately lead to recruitment of GNBP1 and a unique ModSp triggering the activation of the Toll and PPO pathways [377]. Kan *et al.* described a detailed mechanism for the activation of PPO and melanin synthesis in *Tenebrio*. In this model (Fig. 10), the active form of Tm-MSP activates Tm-SPE-activating enzyme zymogen (proSAE) which in turn activates Tm-SPE zymogen (proSPE). Active Tm-SPE subsequently cleaves the Spätzle proprotein (proSpz) into processed Spz which activates the Toll pathway leading to AMP production. Tm-SPE also cleaves Tm-PPO and Tm-cSPH1, resulting in the formation of a melanization complex that ultimately induces local melanin synthesis on the surface of bacteria [378]. In fact, Lee *et al.* revealed that cleavage of cSPH1, also referred to as *Tenebrio* masquerade-like serine proteinase homologue (Tm-mas) is necessary for PO activity and that cSPH1 is a PPO activating cofactor [379]. Interestingly, cSPH1 was found to co-localize with 1,3-β-D-glucan recognition protein (Tm-GRP) that specifically binds to curdlan polymers (β-1,3-glucan fungal polymer) [380]. Perhaps through binding to microbial cell wall components, cSPH1 inhibits PO diffusion to the hemolymph allowing for a localized immune response. Regarding the control of the β-1,3-Glucan-induced melanin synthesis pathway, three novel serpins have been reported to cooperatively regulate this response in *T. molitor*. The three serpins formed specific complexes with three Toll cascade-activating SPs: SPN40 complexed with MSP, SPN55 with SPE-activating enzyme (SAE), and SPN48 with SPE. In addition, there was a significant decrease in melanin synthesis in larvae injected with a mixture of the three serpins and β-1,3-glucan as compared to the control groups. An *in vitro* assay further confirmed that the conversion

of PPO to PO, which is catalyzed by active SPE, is inhibited by SPN48 [381]. Zhao *et al.* reported that a 43-kDa protein, specifically disappeared from the hemolymph of *T. molitor* during melanin synthesis. The protein was found to function as a melanization-inhibiting protein (MIP), specifically inhibiting melanin synthesis, but not PO activity *in vitro*. Furthermore, silencing this gene by RNAi induced a strong melanization reaction *in vivo.* Indeed, further studies are needed to elucidate the molecular mechanism by which MIP manifests its regulation of the melanization reaction [382].

- *Anopheles gambiae*

The hemocyte-specific complement-like protein TEP1 plays a key role as the most upstream activator of the melanization response, so far, in *A. gambiae* mosquitoes (Fig. 10). Blandin *et al.* reported that *TEP1* kd completely abolished *P. berghei* melanization in the refractory strain, thus converting refractory mosquitoes into susceptible [278]. Similarly, silencing *TEP1* together with *CTL4,* a potent inhibitor of the melanization cascade, [156] completely blocked melanization in N'gousso susceptible *A. gambiae* mosquitoes [295]. TEP1 seems to be involved in the activation of the melanization cascade in response to bacterial infections as evident from the strong inhibition of PPO activation in *TEP1* kd mosquitoes relative to ds*LacZ*-injected controls following *E. coli* injections [300]. A systematic, *in vivo* RNAi screen identified two cSPHs, CLIPA8 and CLIPA2, as positive and negative regulators of *P. berghei* melanization in *A. gambiae* mosquitoes. CLIPA8 is required for ookinete melanization in both the R and S strains. Nearly all melanized ookinetes disappeared in R mosquitoes silenced for *CLIPA8.* Similarly, melanization was practically abolished in *CTL4*/*CLIPA8* double knockdown (dkd) S mosquitoes [383]. It has been shown that PPO activation in response to bacterial and fungal infections requires CLIPA8 as well

[311, 384], suggesting that it is acts as a core positive regulator of the melanization response to diverse classes of microorganisms. In contrast to *CLIPA*8 kd, a significant increase in the numbers of melanized ookinetes was reported in *CLIPA2* kd R and S mosquitoes, indicating a negative regulatory role for CLIPA2 in melanization [383]. This phenotype was later shown to be due to an exaggerated TEP1-mediated response in *CLIPA2* kd mosquitoes. In fact, CLIPA2 was also shown to localize to microbial surfaces in a TEP1-dependent manner where it might negatively regulated the conversion of the full-length TEP1-F form into active $TEP1_{cut}$. The enhanced TEP1 consumption observed in *CLIPA2* kd mosquitoes was accompanied with an abnormally high phenoloxidase activity in response to systemic bacterial infections [301]. More recently, SPCLIP1 was found to act as a positive regulator of TEP1 accumulation on malaria parasites and bacteria, and of melanization. *SPCLIP1* kd resulted in a strong decrease in PO activity comparable to that observed in *CLIPA8* kd mosquitoes. Western blot analysis coupled with RNAi revealed that CLIPA8 cleavage following bacterial infections occurs in a SPCLIP1 and TEP1 dependent manner [300]. More recently, another cSPH, CLIPA14, was shown to negatively regulate *P. berghei* melanization in a TEP1-dependent manner. *CLIPA14* kd triggered a potent melanotic response against *P. berghei* ookinetes and an exaggerated hemolymph phenoloxidase activity following systemic bacterial infections [385]. A subsequent study identified CLIPA28 as a novel cSPH essential for the mosquito melanization response. More importantly, this cSPH was shown to be part of a complex cSPH module that exhibits a hierarchical mode of activation with SPCLIP1 being upstream followed by CLIPA8 then CLIPA28. Indeed, TEP1 seems to be in the most upstream position in the melanization response as its depletion abolished the activation of this network after septic infections [311, 383, 384,

386]. The positioning of TEP1 upstream in the melanization cascade reveals the existence of a crosstalk between complement and melanization in mosquitoes. As for CLIPA14, it seemed to be subject to multiple regulations by positive (TEP1, SPCLIP1, CLIPA8 and CLIPA28) and negative (CLIPA2) regulatory cSPHs [385, 386]. Despite the fact that their roles as positive and negative regulators of TEP1 accumulation on microbial surfaces support an upstream position within the cSPH module, the exact positions of SPLCIP1 and CLIPA2 are still putative as their cleavage profiles have not be characterized yet [386]. A master negative regulator of this network is SRPN2 as evident from the strong increase in the cleavage of CLIPA8 and slight reduction in the full-length forms of CLIPA28 and CLIPA14 upon septic infection in *SRPN2* kd mosquitoes. Interestingly, SRPN2 depletion also triggers enhanced network activation in naïve mosquitoes, as manifested by a dramatic reduction in hemolymph levels of all these cSPHs which paralleled that of PPO [386]. SRPN2 probably acts on yet unidentified cSP(s) involved in the proteolytic processing of CLIPA8, CLIPA28 and CLIPA14 [386]. Indeed, CLIPB9 has been shown to be directly inhibited by *A. gambiae* SRPN2 *in vitro* and its kd partially reverts the SRPN2 phenotype *in vivo* [387], however, CLIPB9 does not seem to regulate the cleavage of any of these cSPHs [386]. As with *CLIPB9*, *CLIPB8* kd partially reversed the phenotype induced by silencing *SRPN2*. Nonetheless, SRPN2 did not efficiently inhibit CLIPB8 activity *in vitro* [388]. CLIPB8 also did not seem to regulate the cleavage of these cSPH. Systematic analysis of *A. gambiae* CLIPB and CLIPC members will be needed to identify those CLIPs that regulate the activation cleavage of the key identified cSPHs. In addition to cSPHs, a set of cSPs including CLIPB3, B4, B8, and B17 were also

reported to promote ookinete melanization however the hierarchical order of the mosquito cSP remains to be determined [383].

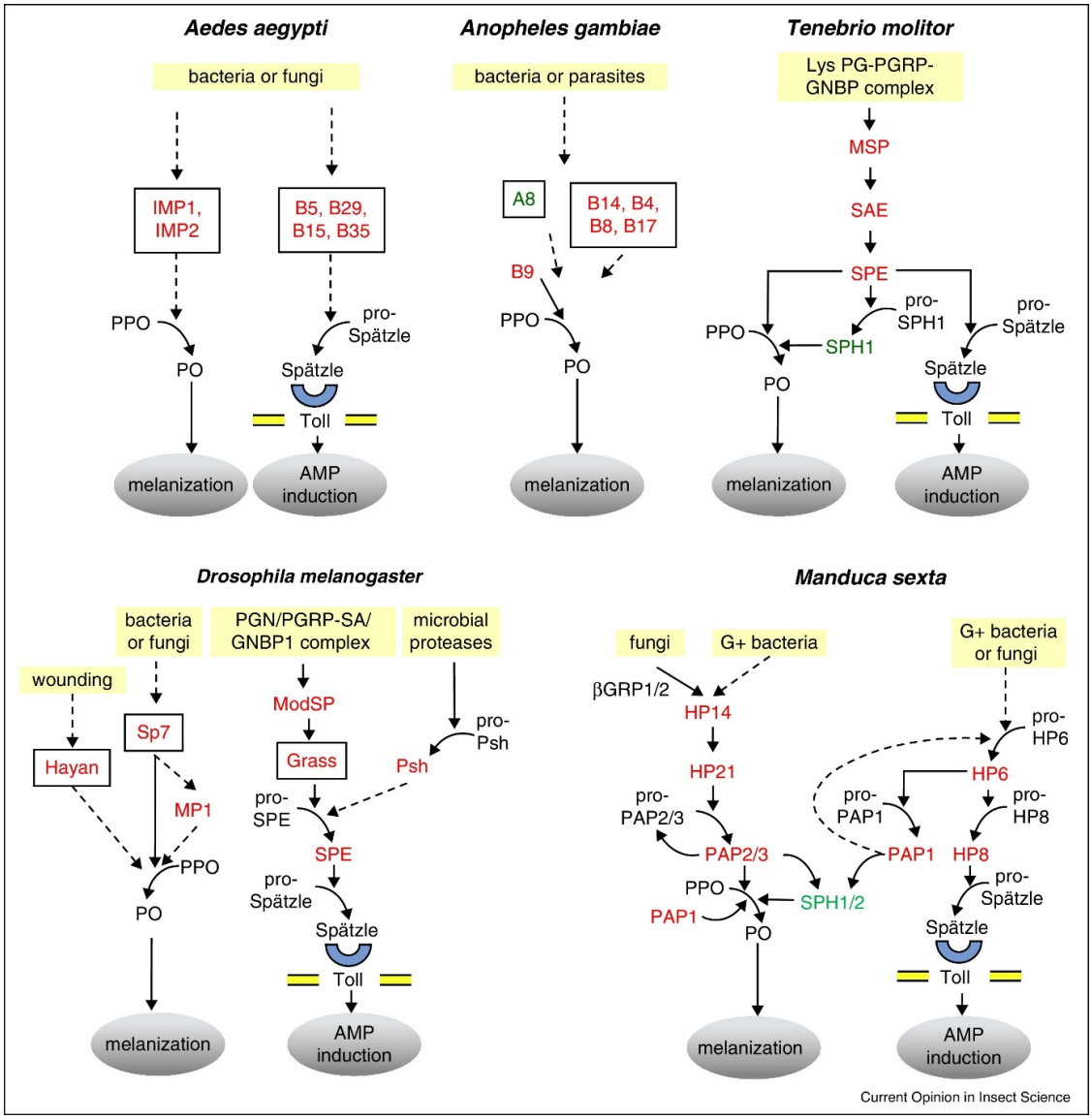


Figure 10. Schematic overview of the protease cascades regulating menaization in model insect species.

CLIP proteases are organized in pathways that result in activation of PPO leading to melanization or of proSpätzle leading to Toll pathway activation. For protease names shown in boxes, the activating protease and the protease's substrate are not yet known despite existing genetic evidence for involvement in an

immune pathway. Dashed arrows indicate putative steps that have not been verified experimentally. (Adapted from [350])

e. Phagocytosis

Phagocytosis is an evolutionarily conserved effector cellular process that is mediated by insect hemocytes in response to infections with several classes of microbes [389-394]. Mosquitoes harbor three populations of hemocytes. Granulocytes constitute the most abundant hemocyte type, comprising 80-95% of the circulating hemocyte population, while each of oenocytoids and prohemocytes account for ≤10% of hemocytes in circulation [105]. In addition to this population of circulating hemocytes, a small group of hemocytes (~25%) in adult mosquitoes are otherwise sessile [395]. According to multiple studies, the total number of hemocytes in circulation is somewhere between 500 and 4,000 [102, 395-402], 95% of which are phagocytic[397]. Phagocytosis is initiated as early as 5 minutes after exposure of a foreign invader. This highly important and complex antimicrobial defense mechanism is regulated by pattern recognition receptors, transmembrane receptors and other signaling proteins. In *A. gambiae*, TEP1 was previously shown to serve as a complement-like opsonin promoting phagocytosis of three Gram-negative bacterial species in a mosquito hemocyte-like cell line. In contrast, the same protein was not sufficient to promote the uptake of Gram-positive *S. aureus* and two other bacterial species [116]. A subsequent *in vivo* study in *A. gambiae* mosquitoes demonstrated that TEP1 plays a very similar role to C3 whereby its kd dramatically decreased the efficiency of phagocytosis of both *E. coli* (~60%) and *S. aureus* (~40%). Similarly, silencing *TEP4* significantly affected phagocytosis of both *E. coli* and *S. aureus* by more than 50%. In addition, *TEP3* depletion inhibited phagocytosis of *E. coli* but not that of *S. aureus*, and *LRIM1* kd

decreased the uptake of *E. coli* (~70%) but not *S. aureus* [310]. The hypervariable immunoglobulin domain-containing receptor AgDscam is another molecule involved in phagocytosis with effects comparable to those shown for TEP1 *in vitro*. In fact, depletion of AgDscam decreased the phagocytic capacity of an immortal hemocyte-like cell line by about 60% and 55% for *E. coli* and *S. aureus*, respectively [142]. Several transmembrane receptors have also been implicated in phagocytosis among which are a ß-integrin (BINT2), peptidoglycan recognition proteins and a low-density lipoprotein receptor-related protein (LRP1) [96]. Moita *et al.* demonstrated the involvement of BINT2 in engulfment of *E. coli* by a hemocyte-like mosquito cell line [403]. The *in vivo* depletion of *BINT2* in *A. gambiae* mosquitoes reduced phagocytosis of *E. coli* by more than 70%. A reduction in *E. coli* uptake by ~60% was also reported upon silencing *PGRPLC* [310]. Moroever, *PGRPLA* kd had a similar, yet less pronounced effect compared to *PGRPLC* kd. Similarly, depletion of *LRP1* decreased uptake of *E. coli* by 80% and that of *S. aureus* by 50% [310]. Recently, a number of novel regulators of phagocytosis in *A. gambiae* were identified of which we mention FBN8 and FBN9. While silencing *FBN8* decreased phagocytosis of *E. coli* bioparticles, that of *FBN9* had the opposite effect [404]. Lastly, the intracellular cell death proteins (CED2, 5, and 6) were shown to play a role in internalization of bacteria in *A. gambiae* mosquitoes. In fact, CED6-like (*CED6L*) kd caused a substantial reduction in phagocytosis efficacy of both *E. coli* and *S. aureus*. The knockdowns of *CED2L* and *CED5L* also decreased phagocytosis of both types of bacteria. Interestingly, the effects of CED6L and CED5L seemed to be additive suggesting that the two genes function in two different pathways to regulate phagocytosis. Indeed, epistasis analysis confirmed the existence of two major phagocytic pathways that regulate phagocytosis of bacteria

in *A. gambiae* (Fig. 11) : TEP1, TEP3, LRIM1 and LRP1 all group in the CED6 pathway whereas TEP4 and BINT2 are genetically associated with the CED2/CED5 pathway. Furthermore, both *E. coli* and *S. aureus* were shown to accumulate in *CED5L*/*CED6L* kd mosquitoes to levels up to three times higher than those in the control group, an effect most likely due to inhibition of phagocytosis. The aforementioned study also revealed that the inactivation of 26 genes changes phagocytosis of at least one bacterial species by 45% or more. In addition to genes encoding putative transmembrane receptors and hemolymph circulating molecules, the group included miscellaneous genes, enzyme-encoding genes, as well as genes encoding chaperone-like molecules and protein-protein interaction domains [310]. A recent study in *A. gambiae* reported a novel role of cytoplasmic actin 5C as an extracellular PRR that is secreted upon immune challenge and binds with high affinity to bacteria mediating their phagocytosis [405]. It is noteworthy to mention that phagocytosis is not involved in ookinete killing or in the clearance of dead parasites. This is expected given the fact that ookinete killing occurs at a location where a direct contact between parasites and hemocytes is lacking due to the presence of the basal lamina. Exceptionally, such direct interaction was observed on one occasion, when the integrity of the basal lamina appeared to be breached [406].

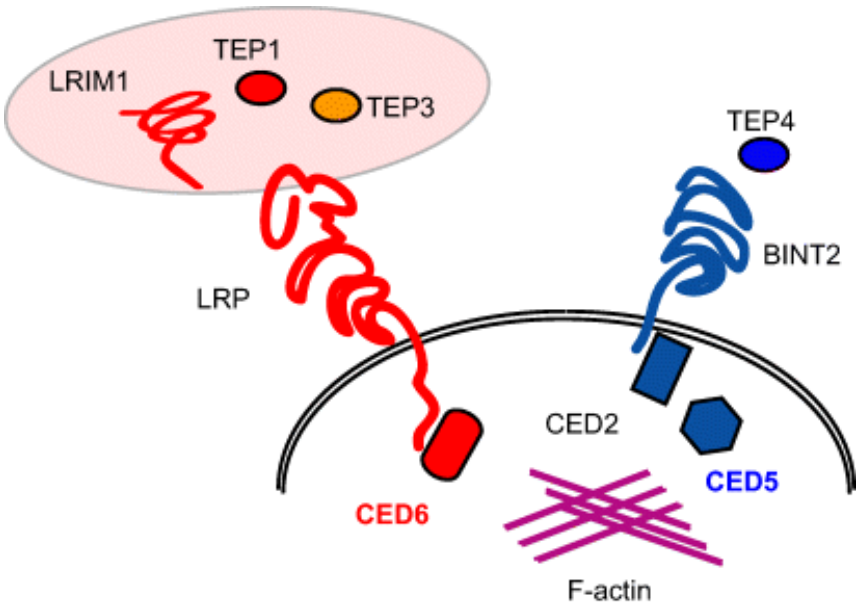


Figure 11. Phagocytosis in mosquito immune responses.

Phagocytosis of bacteria in *A. gambiae* is regulated by two major pathways. These regulators were assigned to one or the other pathway by the simultaneous silencing of a representative of each pathway, CED5 or CED6, with identified regulators of phagocytosis in mosquitoes. Proteins involved in anti-parasitic responses are shaded in pink. (Adapted from [406])

f. Cellular immune response to invading ookinetes: The Actin hood

Another cellular immune response, yet poorly understood, is the midgut epithelial response to invading ookinetes. Early studies in *A. gambiae* reported an organelle-free zone consisting of finely granular and filamentous material that encircled dead or dying *Plasmodium gallinaceum* (*P. gallinaceum*) ookinetes in the basal lamina of the mosquito midgut [407]. This granular zone was previously observed in *P. gallinaceum*-infected *Ae. aegypti* [408]. A more recent study by Vlachou *et al.* reported a 'hood' of lamellipodial origin that covers the ookinete during its egress from the midgut epithelium. The 'hood' is formed by a single, more or less tubular

lamellipodium of the invaded cell itself that is enriched with fibrillary material most probably representing actin filaments. Similar observations were described in both *A. gambiae* and *A. stephensi* [409]. Indeed, genome-wide expression profiling of *A. gambiae* midguts infected with *P. berghei* revealed that the largest class of invasion-upregulated genes corresponded to players in actin- and microtubule-cytoskeleton remodeling. Functional genetic analysis by RNAi further revealed that the intracellular local activator of actin cytoskeleton dynamics Wiskott–Aldrich syndrome protein (WASP) acts as an antagonist of *P. berghei* and *P. falciparum* development [410, 411]. However, it remains unclear whether a 'hood' forms around *P. falciparum* ookinetes as well. In contrast, an independent study did not reveal a role for this actin hood in parasite killing. Using a combination of transmission electron microscopy and immunofluorescence, Shiao *et al.* identified an organelle-free actin zone formed within the basal cytoplasm of midgut epithelial cells that surrounded dead ookinetes that were almost invariably extracellular [307]. This zone was formed by several apparently healthy epithelial cells [307] rather than formed as a parasite exited a damaged midgut cell as previously reported by Vlachou *et al*. [409]. Moreover, knockdown of frizzled-2 (*Fz2*) and cell division cycle 42 (*Cdc42*), which both control cytoskeletal rearrangements reduced the formation of an organelle-free actin zone (AZ) but did not affect parasite survival demonstrating that AZ formation doesn't necessarily constitute an effector cellular response against invading ookinetes [307]. While AZ formation is dispensable for parasite killing by lysis it seems to regulate the melanization response against *Plasmodium* parasites in refractory mosquito backgrounds [307]. Further studies using different mosquito strains and *Plasmodium* species will be required to define

accurately the role of this actin zone in the midgut epithelial response to *Plasmodium* ookinetes.

## C. Immune defense against *Serratia marcescens* intestinal infections

### *1. Serratia marcescens as a model bacteria to study gut immunity*

a. Characteristics of *Serratia*:

Bacteria of the genus *Serratia* are Gram-negative rods that are facultative anaerobes. The genus comprises 10 species ubiquitously distributed in soil, sediments, water, plant roots, on animal epithelial surfaces as well as in the gastrointestinal tract of animals [412]. These bacteria have also been reported to live in symbiosis with and cause disease to various economically important insects and nematodes [413-416]. As for mosquitoes, among the dynamic microbial community composed of bacteria, viruses and fungi that has been reported to colonize the gut, salivary glands and reproductive organs [417-419], *Serratia* is indeed a prevalent member. In fact, *Serratia* was reported to be among the abundant genera of the gut microbiota in *A. gambiae* mosquitoes from Kenya [417]. Moreover, in a study exploring the composition of microbiota in lab reared *A. gambiae* mosquitoes, it was demonstrated that *Serratia* sp. was a dominant member within five consecutive generations [127]. Seitz *et al.* reported large numbers of *Serratia marcescens* (*S. marcescens*) in the midguts of adult lab-reared *A. stephensi* as well [420]. *S. marcescens* was also found to be dominant in the midguts of field-caught *A. stephensi* larvae and adult females [421]. Furthermore, *S. marcescens* and *Serratia* sp. were isolated from *A. albimanus* caught in southern Mexico [422]. In addition to anophelines, *Serratia* sp. were predominant among bacterial isolates from

adult *Ae. aegypti* guts [423]. *S. marcescens* was also among the most frequently isolated species from midguts of wild-caught *Culex pipiens* (*C. pipiens*) mosquitoes [424], indicating that *Serratia* can colonize several mosquito species. Despite being frequent residents of the midgut microbiota of mosquitoes, certain *Serratia* species have the ability to cause pathogenesis by encoding various virulence factors such as flagella, lipooligosaccharide (LOS), iron uptake and transportation systems, serralysins, hemolysins, and chitinases [425]. Another characteristic of *Serratia* is its ability to form biofilms, a phenomenon that in *Serratia* is controlled by quorum sensing, type 1 fimbriae, and carbon and nitrogen sources [426, 427].

b. The effect of *Serratia* on vector competence:

*Serratia* has been reported to influence mosquito-pathogen interactions, and consequently vector competence. In fact, co-infection with *Serratia odorifera*, a midgut inhabitant of field collected and laboratory reared larvae and adult *Ae. aegypti* females, was shown to enhance susceptibility to Dengue-2 Virus [428]. On the other hand, a *S. marcescens* isolate displayed a strong ability to colonize and persist in *A. gambiae* mosquito midguts and to block *Plasmodium* development *in vivo* and *in vitro* [429]. Systemic bacterial infection with this isolate compromised mosquito survival, however, the mortality rate was higher when similar numbers of bacteria were introduced into the mosquito's midgut via blood feeding. The *S. marcescens* bacteria free culture supernatant inhibited *P. berghei* ookinete development *in vitro* as well as the asexual stages of *P. falciparum*, suggesting that a bacteria-secreted soluble factor is mediating the observed anti-*Plasmodium* effect in the mosquito [429]. In another study, the percentage of female *A. albimanus* mosquitoes infected with *P. vivax* decreased from 71% when *S. marcescens* was excluded from the blood meal to only 1% when *S.*

*marcescens* was simultaneously introduced per os in the blood meal [422]. Bai *et al.* identified a mosquito *S. marcescens* strain (*Serratia* Y1) in the midgut of *A. stephensi* that, when introduced through the oral route, inhibits *P. berghei* infection through activation of the mosquito Toll immune pathway. Silencing *Serratia* Y1-induced anti-*Plasmodium* factors including, TEP1, FBN9 or leucine-rich repeat domain protein family 7 (LRRD7) rescued the refractoriness conferred by *Serratia* Y1 infection [430]. *S. marcescens* can inhibit parasite development in the mosquito midgut in a manner independent of the induction of anti-*Plasmodium* immune responses. In this context, *S. marcescens* introduced into *A. stephensi* mosquitoes through oral feeding exerted anti-*Plasmodium* activity that was not immune-mediated but rather correlated with phenotypic variation in the bacterial cellular and structural features. The authors suggested a scenario whereby *S. marcescens* directly interacts with the parasite interrupting ookinete invasion of the midgut [431]. Yet another mean by which *Serratia* could exert its anti-*Plasmodium* effect is through a bacterium-produced inhibitory metabolite, prodigiosin [432, 433]. In addition to *Plasmodium*, *S. marcescens* has been also shown to mediate the inhibition of other parasites in *vitro* including, *Leishmania* [434] and *Trypanosoma* [435].

### c. Immunity to oral *Serratia* infections in invertebrate models

#### *i*. *Caenorhabditis elegans*

*S. marcescens* is capable of establishing an intestinal infection in *Caenorhabditis elegans* (*C. elegans*) in less than 6 hrs following transfer of worms to a lawn of *S. marcescens* strain Db11, as evident from intact bacteria found within the intestinal lumen. There, bacteria proliferated rapidly causing a progressive distension of

the intestinal lumen that ultimately led to destruction of the worm's intestinal epithelium and death of infected worms after 72 hrs of contact with Db11 [436]. Using high-density copy DNA (cDNA) arrays, a group of genes were identified that are robustly induced upon infection by the Gram-negative bacterium. Among these, seven genes showed a greater than 2-fold induction at both 24 and 48 hrs, of which three encode lectin domain-containing proteins with no clear homologs in other species, and only one encodes a protein with overall similarity to vertebrate gastric lipases. Transcription of lysozyme genes *lys-1, lys-7*, and *lys-8* was markedly elevated following infection, despite not falling within the strict selection criteria applied to identify the seven genes. Indeed, lysozymes, whether acting independently or synergistically with antimicrobial peptides/proteins, play an important role in antibacterial defense. Expression of all three genes was mainly detected in the worm's intestinal cells, perhaps due to the fact that *S. marcescens* remains confined to the intestinal lumen during infection. As for the lys-1 protein, it was found to be localized within vesicles that appeared to be concentrated toward the apical surface of intestinal cells. Longevity of worms overexpressing *lys-1* in the presence of Db11 was not significantly different from that of control worms. However, overexpression of *lys-1* was shown to augment the resistance of *C. elegans* to Db1140, a strain of *S. marcescens* that is less virulent than Db11. Db1140 is a protease-deficient derivative of Db11 that is otherwise well known to secrete extracellular bacterial proteases [437]. As such, one possible scenario is that once secreted from vesicles into the intestinal lumen, lys-1 can protect worms from Db1140. On the other hand, this effect is countered by the secretion of bacterial proteases in case of infections with Db11 [436]. Certain infection-inducible genes, among which is *lys-8,* were previously identified in a study looking at genes controlled by the transforming

growth factor-β (TGFβ)-related gene *dbl-1* [438]. Indeed, *dbl-1* mutants exhibited a dramatically reduced tolerance to infections with both Db11 and Db1140 relative to wild-type worms [436]. A separate study by Kurz *et al.* revealed that wild-type worms become visibly sick 2 days after transfer from the standard *E. coli* strain OP50 to Db11 lawns, eventually succumbing to the infection. To address whether death is via toxin-mediated mechanisms, worms were transferred to heat-killed Db11 in the presence or absence of supernatants from saturated Db11 cultures. Interestingly, these worms survived as long as the worms that fed on OP50 did, and showed no signs of sickness, excluding the possibility that a toxin is responsible for Db11-mediated killing [439]. Instead, a contact for around 30 hrs between live Db11 and the worms was needed to compromise survival. The phenotype can otherwise be explained by an incapacity of OP50 to colonize the intestine. In fact, while OP50 bacteria were broken down by the grinder located in the terminal bulb of the pharynx and as such no intact bacteria made it to the intestine, intact Db11 bacteria were seen to accumulate in the lumen of the intestine as soon as 2 hrs after transfer from OP50. The authors suggested that Db11 might be capable of disrupting the function of the grinder as brief feeding on Db11 before being transferred to OP50-GFP was sufficient to allow some intact OP50 bacteria to pass the grinder. The symptoms of infection of *C. elegans* with *S. marcescens* included, a progressive distension of the intestinal lumen 24 hrs post contact with Db11 that was concurrent with exponential bacterial growth and a progressive vacuolation of the intestinal cells accompanied by a decrease in the volume of the worm's intestinal epithelium. Using this system the authors also identified a number of bacterial genes necessary for the full *in vivo* virulence of Db11including, those that function in LPS biosynthesis, iron uptake and hemolysin production [439].

*ii*. *Drosophila melanogaster*

Nehme *et al.* established a model of intestinal infection of *D. melanogaster* with *S. marcescens* through oral feeding. As opposed to septic injury, which killed flies in a day following direct injection of the bacteria into the body cavity of *Drosophila,* flies succumbed to infection only after 6 days from feeding on *S. marcescens* DB11 [440]. This slow killing rate could not be attributed to the confinement of the bacteria to the digestive tract, since *S. marcescens* was recovered from the hemolymph of infected flies, demonstrating its ability to rapidly escape from the digestive tract into the internal hemocoel of the host. Interestingly, electron microscopy revealed that *S. marcescens* is found within vacuoles in the midgut epithelium. Despite the presence of numerous small electron-translucent vacuoles in the cytoplasm of invaded cells that indicate an important cellular stress, the cells did not exhibit any of the hallmarks of apoptosis or necrosis. Furthermore, ingested *S. marcescens* failed to elicit a systemic immune response, although Imd pathway mutants were more susceptible to *S. marcescens* oral infections as compared to wild- type flies. A local immune response in the midgut manifested by the induction of Diptericin transcription accounted for the role of the Imd pathway in host defense. On the contrary, the Imd pathway did not protect the fly effectively against *S. marcescens* in the septic injury model. The contribution of phagocytosis to host defense against orally introduced *S. marcescens* was also evaluated. Flies in which this cellular response was blocked exhibited increased susceptibility to *S. marcescens* oral infections, indicating that phagocytosis plays a vital role in defense against ingested *S. marcescens* that have escaped into the hemolymph. Interestingly, the authors reported that a systemic immune response is elicited by ingested bacteria that reach the hemolymph only when phagocytosis is blocked, in

support of a model wherein, peptidoglycan fragments released during bacterial growth activate the Imd pathway. As opposed to the oral infection model, *S. marcescens* is not sensitive to phagocytosis in the septic injury model, possibly due to the distinct virulence properties that *S. marcescens* displays upon ingestion as opposed to direct injection [440].

*iii*. *Anopheles gambiae*

Stathopoulos *et al.* utilized single-nucleotide polymorphism (SNP) genotyping to identify genetic variations associated with the outcome of oral *S. marcescens* infection in *A. gambiae* mosquitoes. The array identified 138 genes associated with the outcome of infection including those encoding PGRPLC, which is well known to recognize peptidoglycan and activate the Imd/Rel2 signaling pathway [115, 196, 197], the epidermal growth factor receptor EGFR, and a set of three type III fibronectin domain proteins (FN3Ds) [441]. RNAi-mediated silencing of these identified candidate genes increased *Serratia* levels in the guts of orally infected mosquitoes and altered the bacterial population structure of the mosquito gut in favor of *Enterobacteriaceae.* Other genes that were also associated with *S. marcescens* infection phenotype included those encoding, putative transcription factors with homeobox-like or DNA-binding domains, alpha-glucosidase and alpha-mannosidase homologs, EGFR, CLIPE6 and CLIPE7, two Toll-like receptors, a protein with a ricin B lectin domain, five annotated or putative GPCRs and two gustatory receptors, Gr9 and Gr10. In a manner similar to FN3Ds, mosquitoes silenced for *Gr9* harbored a bacterial load in the gut that is 36 to 48-fold more than *dsLacZ* treated controls, indicating that Gr9 plays an antibacterial role that influences the outcome of *S. marcescens* intestinal infection. In this same study, DNA microarrays were also used to monitor transcriptional profiles of mosquito guts

following *S. marcescens* infection. This analysis revealed 97 differentially regulated genes whose enrichment analysis identified 16 gene ontology (GO) terms that were significantly overrepresented, most of which were related to two functional classes: serine-type endopeptidases and chitin-binding genes [441].

### *2. Local immune defenses in the mosquito gut to intestinal pathogens*

The peritrophic matrix (PM) is a semipermeable extracellular layer composed mainly of chitin, proteins, and proteoglycans that lines the midgut of most insects. There exists two types of PM, type 1 (PM1) and type 2 (PM2). While PM1 formation is stimulated within minutes of blood meal ingestion in hematophagous insects, PM2 is constitutively secreted independent of the feeding status of the insect by cardia, a specialized organ located at the junction of the cuticle-lined foregut and midgut. Mosquitoes secrete a PM2 during larval life and a PM1 during adult life (reviewed in [442]). Among the functions attributed to the PM are protection from pathogens, abrasion, toxic compounds, and in certain cases facilitating digestion. Indeed, the study of *Plasmodium* life cycle in the mosquito offers the best evidence for a role of type 1 PM as a barrier to pathogen invasion. Huber *et al.* reported the presence of a malaria parasite chitinase detectable 15 hrs after zygote formation, which is the time required for maturation of the zygote to an invasive ookinete. This in addition to the presence of chitin in the peritrophic membrane and the fact that the peritrophic membrane is disrupted during invasion led the authors to conclude that the chitinase secreted by ookinetes allows them to "punch a hole" through the chitin-containing PM [443]. Massive bacterial proliferation in the gut after blood feeding [127] was shown to upregulate expression of genes encoding PM proteins such as glucosamine-fructose-6-

phosphate aminotransferase (GFAT) and chitin synthase enzyme 1 (CHS1) [444]. It was also reported that PM plays a role in limiting the growth and persistence of *Enterobacteriaceae* within the gut, thus preventing this family of bacteria from invading the mosquito body cavity and seeding a systemic infection. In addition, this structure was shown to promote restoration of gut homeostasis through facilitating the efficient clearance of bacteria from the gut after blood bolus digestion [444].

Another physical barrier described in *A. gambiae* mosquitoes is the network of dityrosine-linked proteins observed on the luminal surface of the midgut epithelium in blood-fed mosquitoes. A heme peroxidase, IMPer, is secreted by *A. gambiae* midgut epithelial cells upon blood feeding and together with Duox, catalyzes protein crosslinking in the mucin layer occupying the ectoperitrophic space between the PM and the midgut epithelium. The IMPer/Duox system mediates protein dityrosine crosslinking forming a network that decreases gut permeability to immune elicitors, thus limiting their interaction with midgut epithelial cells and the subsequent activation of a local immune response. This offers an advantage to the microbiota but at the same time provides a suitable environment for malaria parasites to flourish within the midgut lumen without inducing NOS expression. Disruption of this barrier through silencing IMPer or Duox drastically reduces *Plasmodium* infection indicating that IMPer and Duox are required to prevent activation of midgut responses to *Plasmodium* [330].

In *Drosophila*, Duox has been shown to mediate a microbicidal response that prevents overproliferation of dietary bacteria and yeast [445, 446]. One attractive possibility that is yet to be validated would be that a similar barrier is formed in *Drosophila.* In fact, immune-regulated catalase (IRC), the amino acid sequence of which bears a high degree of similarity to various kinds of heme-containing peroxidase,

may be involved in this process. Silencing IRC was shown to induce high mortality in flies fed on microbe contaminated food. Furthermore, this mortality could be rescued by treating flies with antioxidants, further confirming that high levels of ROS are behind this observed mortality [447].

A critical immune modulator during intestinal infections is the gut microbiota constituted of a vast and intricate microbial community. In *Anopheles*, this community is dominated by *γ-Proteobacteria* and in particular*, Enterobacter, Serratia, Pantoea, Asaia, Aeromonas, Pseudomonas,* and *Bacillus* [52, 421, 448-451]. The microbiota interferes with *Plasmodium* development in the mosquito gut either directly or indirectly as described in several reports [127, 336, 422, 429, 452]. The intracellular maternally transmitted bacteria *Wolbachia* has been shown to potentiate *Plasmodium* and filarial parasite killing in anopheline mosquitoes through induction of ROS [51, 453]. *Enterobacter* isolates from Zambian *A. gambiae* exhibited direct plasmodicidal activity against *P. falciparum* in the midgut. This anti-plasmodial effect was attributed to direct inhibition by the bacteria-produced ROS [336]. In addition, *P. falciparum* ookinete development was also shown to be inhibited by soluble factors released by *S. marcescens* in *Anopheles* mosquitoes [429]. Interestingly, *A. gambiae* mosquitoes harboring an intact microbial flora exhibit higher basal expression levels of immunity genes compared to antibiotic-treated controls. These genes encode AMPs, signal-transducing serine proteases, Imd pathway components, fibrinogen-related and thioester-containing proteins among others [127], indicating that the microbiota primes the mosquito immune response. Moreover, the microbiota has been shown to activate the Imd pathway through the receptor protein PGRP-LC, a pathway known to defend mosquitoes against *P. falciparum* infection [124, 127, 184].

Parasites that escape the PM and withstand the hostile environment brought upon by the microbial community residing in the gut are subject to other effector responses in the gut epithelium. As they traverse the midgut epithelium, ookinetes undergo epithelial nitration which promotes the eventual killing of ookinetes by TEP1 mediated lysis [17, 277]. Epithelial cells produce NO [262, 454], and ROS [454] to limit *Plasmodium* survival. Microarray studies revealed several effector molecules from diverse gene families that respond to *Plasmodium* ookinete invasion of the midgut. For instance, in one study, 45 genes were induced in the midgut of *A. gambiae* mosquitoes by *P. falciparum* and 29 genes by *P. berghei* ookinete invasion [139]. The two groups exhibited similar functional distribution whereby almost half of the genes encoded putative pattern recognition receptors that belong to the MD2-like protein family (AgMDL), FBN, the thioester-containing protein family (Tep), the GNBP family, the PGRP family , the C-type lectin family (CTL), gal-lectin family (GALE), the scavenger receptor family, LRRD, and the bacteria recognition family (AGBP) [111, 116, 122, 123, 319, 455-459]. Other genes included those encoding immunity-related serine proteases and serine protease inhibitors, enzymes involved in melanization reactions, as well as lysozymes and the mosquito-specific antimicrobial peptide gambicin. In fact, the antimicrobial peptide gambicin was previously shown to be induced locally in the midgut of *A. gambiae* mosquitoes during early and late stages of natural malaria infection [182]. Additional infection-responsive markers expressed in the midgut of adult female *A. gambiae* include, immune-related serine protease-like sequence 5 (ISPL5), the immune-related serine protease 13 (ISP13), the putative infection-responsive galactose lectin (IGALE20), defensin, and AgGNBP [319]. GNBPB4 was also found to weakly co-localize with *P. berghei* ookinetes in the mosquito midgut

epithelium [133]. Another putative pattern recognition receptor, the *AgDscam* gene, was shown to respond to *Plasmodium* invasion of the midgut epithelium by changing its transcript exon repertoires in the infected midguts [142]. The mosquito gut transcriptome is not only modulated by *Plasmodium* parasites but also responds to bacterial infections. For instance, transcriptional profiling of *A. gambiae* mosquito guts 3 days post oral infection with *S. marcescens* identified several differentially expressed genes known to be involved in mosquito immunity. These include PGRPLC, the complement factor regulator LRIM1, serine-type endopeptidases and chitin-binding genes [441].

### *3. Systemic immune priming by the mosquito midgut*

Ookinete invasion of the midgut epithelium brings the gut microbiota into direct contact with midgut epithelial cells. This triggers a long-lived response characterized by a quantitative and qualitative differentiation of hemocytes that allows mosquitoes to mount a more effective immune response to subsequent *Plasmodium* infections [460]. Ramirez *et al.* demonstrated that primed mosquitoes constitutively release a soluble hemocyte differentiation factor (HDF) into their hemolymph. HDF is a complex of Lipoxin A4, a signaling eicosanoid, and Evokin, a lipid carrier of the lipocalin protein family [461]. Recently, the nature of the chemotactic signal that attracts hemocytes to the basal surface of *A. gambiae* midgut cells and establishes immune priming was identified as prostaglandin E2 (PGE2) [462] (Fig. 12). Another form of immune priming was also described in *A. gambiae* mosquitoes in response to *Plasmodium* infection. Injury inflicted by ookinete invasion is detected by hemocytes that come in close contact with the basal surface of the midgut that becomes nitrated by the concerted activity of NADPH oxidase 5 and heme peroxidase 2 [277]. Contact of

hemocytes with the nitrated midgut basal surface triggers local release of hemocyte-derived microvesicles (HdMv) into the basal labyrinth. These vesicles are necessary to mount an effective antiplasmodial response possibly through delivering some critical factor(s) that promote activation of TEP-1, a key component of the mosquito complement-like system [463] (Fig. 13).

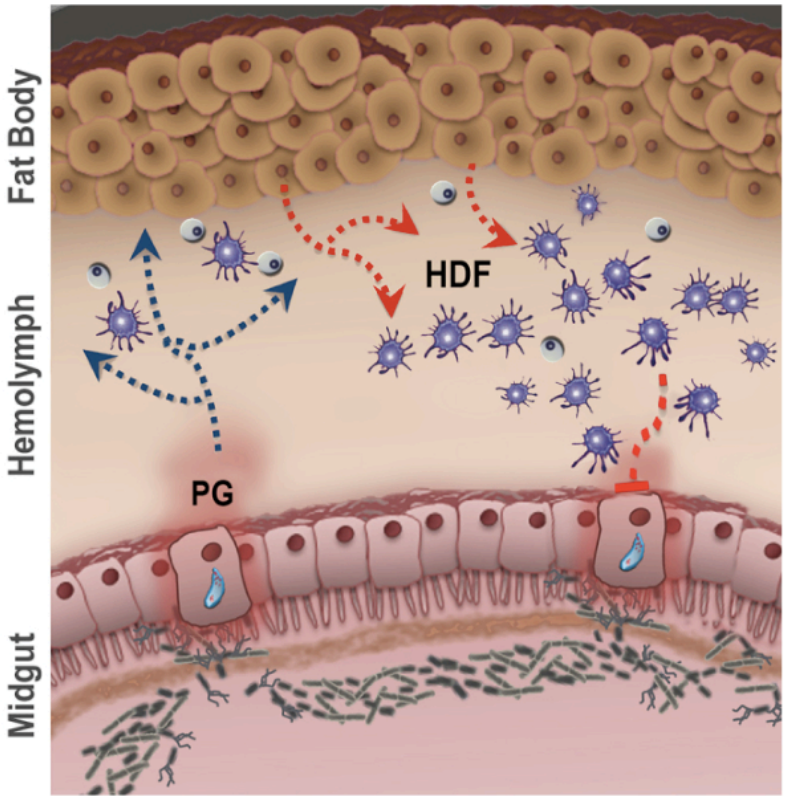


Figure 12. Mosquito Midgut Prostaglandin Release Establishes Systemic Immune Priming.

Priming is established when ookinetes breach the midgut barrier allowing contact of the gut microbiota with epithelial cells. This event is followed by a systemic release of HDF, possibly by the fat body, which induces a permanent increase in the proportion of circulating granulocytes. These are attracted to the midgut surface by PGE2 (Adapted from [462]).

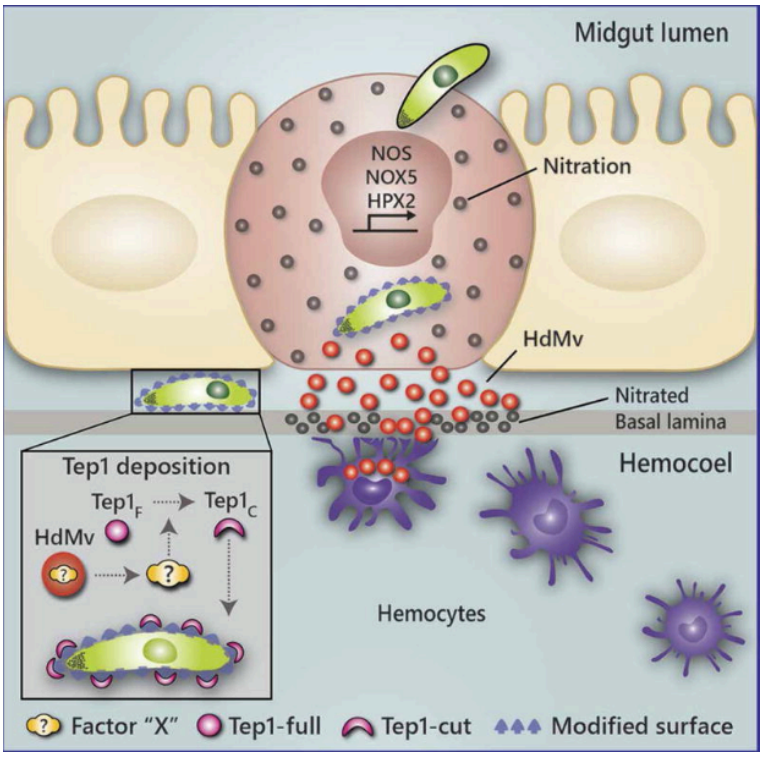


Figure 13. Proposed model of the role of HdMv on mosquito complement activation.

Epithelial nitration is induced in ookinete-invaded cells and involves NOS, NOX5, and HPX2. Hemocytes are attracted to and come in close contact with the nitrated surface of the basal lamina. This triggers release of HdMv that may contain a factor or factors (such as a convertase and/or a convertase cofactor) that promote cleavage of the full-length TEP1 (TEP1-full) to generate TEP1-cut that deposits on the parasite's surface [278] (Adapted from [463]).

Immune priming is not restricted to mosquitoes and has been described in other insects such as *Drosophila*. Ayyaz *et al.* demonstrated that macrophage-like hemocytes control stem cell activity in the *Drosophila* intestine. More specifically, hemocytes are recruited to the intestine upon tissue damage where they secrete the TGFβ/BMP homologue Decapentaplegic (Dpp) which in turn induces intestinal stem cell (ISC) proliferation through Type I receptor Saxophone (Sax)-mediated activation of the Smad

homologue Smox. To re-establish ISC quiescence, activated ISCs then switch their response to Dpp by inducing expression of Thickveins, a second Type I receptor. This interaction between hemocytes and ISCs promotes tolerance against enteropathogens [464]. Another study revealed a similar role for hemocytes in the remote control of intestinal stem cells. Upon injury, hemocytes were shown to release a molecule called Upd3 into the hemolymph which together with Upd2 activates the JAK/STAT pathway in the fat body and the gut. Interestingly, Upd3 released from hemocytes can remotely stimulate stem cell proliferation and an anti-microbial response in the intestine manifested by the expression of Drosomycin-like genes [465].

In *Ae. aegypti* mosquitoes, intestinal epithelium proliferation is induced upon exposure to stress including, oral infection with pathogenic bacteria, following inhibition of PM formation that increases ROS production by the epithelial cells in response to contact with the resident microbiota, and in response to *Dengue* infection. More importantly, the effectiveness of midgut cellular renewal during viral infection is an important determinant of mosquito vectorial competence. In fact, impairment of the Delta/Notch signaling that is involved in cell division and differentiation increases the susceptibility of the refractory strains to DENV infection of the midgut [466]. It is noteworthy to mention that different mosquitoes could possess different number of ISCs in their reservoir. In fact, Janeh *et al.* reported major differences in gut physiological responses between different mosquito species [467]. While no mitotic cells were detected in *A. gambiae* midguts, dividing cells were detected in the midgut of *C. pipiens* that proliferate after bacterial or chemical damage, similar to what was previously observed in *Aedes albopictus* (*Ae. albopictus*) [468] and *Ae. aegypti* [466].

# CHAPTER II

# AIMS OF THE PROJECT

Insects deploy several humoral and cellular innate immune effector mechanisms to clear bacterial infections. While AMPs [469, 470], melanization [471, 472], phagocytosis [473, 474] and complement-mediated attack [475, 476] are often described as the main players in different contexts, several knowledge gaps remain as to their regulation, specificity and relative contribution to microbial clearance. This is further complicated by the fact that the vast majority of bacterial challenges in model insects have been established through an artificial route, by pricking the cuticle to introduce the microbes directly into the hemocoel [310, 321, 391, 477]. While this route of infection has allowed the dissection of systemic anti-microbial immune responses at different levels, it is associated with two major pitfalls: First, microbes are often introduced at large numbers to trigger pathogenesis, which might blur the readouts from distinct effector programs due to saturation effects; low dose infections are most likely the norm in field conditions. For instance, it was recently shown that when a low dose of *S. aureus* is injected into *Drosophila*, the melanization response but not hemocytes or Toll effectors plays a significant role in resisting the infection, whereas, at higher doses the role of hemocytes becomes predominant over that of melanization [355]. Second, this route of infection may not allow sufficient priming of the systemic response whether humoral or cellular. For instance, in the malaria vector *A. gambiae*, invasion of the midgut epithelium by *Plasmodium* ookinetes triggers the release of a hemocyte differentiation factor, constituted of a lipoxin/lipocalin complex, into the hemolymph,

which induces immune priming, preparing the host for a subsequent challenge. Lipocalin is produced by the abdominal wall, possibly in response to unknown signals originating from the invaded midgut [461]. Also, *Plasmodium* midgut invasion triggers the nitration of the basal surface of the midgut epithelium, which upon contact with hemocytes induces the release of hemocyte-derived microvesicles that activate the complement-mediated attack against invading parasites, through unknown factors they deliver [463]. These studies inform that midgut invasion seems to trigger different forms of innate immune priming which might not occur if this route is bypassed.

A large number of functional genetic studies in *A. gambiae* identified several immunity genes with roles in systemic antibacterial defense [116, 124, 133, 138, 139, 142, 155, 310, 478]. However, since bacterial challenges in these studies were performed by cuticle pricking it remains unclear whether these genes significantly contribute to immune defense against systemic infections established through the oral route, i.e., after midgut invasion. This is particularly important since a previous study in *Drosophila* revealed that the virulent *Serratia marcescens* (*Sm*) Db11 strain is resistant to the Imd-mediated immune response during septic infections but is susceptible to the local Imd response in the gut after oral infections [440]. *Sm* is a Gram-negative bacterium with a broad host range including plants, vertebrates and invertebrates [479], and an opportunistic pathogen to vertebrates [480, 481] and invertebrates [440, 482]. Its ability to efficiently colonize the midguts of insects [440, 441] and to invade the midgut epithelium reaching into the hemolymph [440] makes it an attractive microbe to address whether the route of infection alters the contribution of key immunity genes to systemic immune responses. Furthermore, *Sm* is one of the bacterial species identified frequently as member of the microbiota in lab and field-collected *A. gambiae*

mosquitoes [418, 429, 431], which makes it more relevant to studying host-parasite interactions in this important malaria vector. Certain isolates of *Sm* compromised *Plasmodium* development when introduced into the midgut through a blood or sugar meal, most likely due to certain virulence factors released by the bacteria [429, 431]. However, the physiological relevance of *Serratia* symbiosis in insects remains poorly characterized. Here, we chose *A. gambiae* CTL4 and TEP1 which exhibit prominent roles in defense against systemic Gram-negative bacterial infections [116, 139, 155, 310] to determine whether the contribution of immune genes to mosquito resistance to *Sm* infections varies with the route of infection (oral versus injection). TEP1 and CTL4 are required for the clearance of *E. coli* systemic infections [116, 139, 155, 310], however, the fact that *E. coli* is not pathogenic to mosquitoes, and that susceptibility studies require the injection of large numbers of bacteria (approximately 150,000 colony forming units (CFUs) [155]) into the hemolymph raises legitimate questions concerning the significance of the immune contribution of these genes using this bacterial infection model and route of infection. To clarify this situation, we used the virulent *Sm* DB11 bacterial strain that kills mosquitoes at much lower CFUs than *E. coli*, to assess the true contribution of CTL4 and TEP1 to antibacterial defense. We also used RNA sequencing (RNA-seq) analysis to determine whether the different routes of infection are associated with distinct transcriptional responses in the midguts and abdomens of infected mosquitoes.

**Specific aims**

*Specific Aim 1: Investigate whether the route of infection alters the contribution of the key immunity genes,* TEP1 *and* CTL4*, to defense against Sm infections*

1.1. Establish a model for oral feeding of *Sm* in *A. gambiae mosquitoes*

1.2. Compare the tolerance and resistance of *TEP1* kd and *CTL4* kd mosquitoes to oral *Sm* infections versus systemic infections by microinjection

1.3. Determine whether invasion of the midgut epithelium alters the fitness of *Sm* in *CTL4* kd mosquitoes

*Specific Aim 2: Examine the contribution of* Rel2 *pathway and phagocytosis in defense against Sm systemic infections established after gut invasion*

2.1. Compare the tolerance and resistance of *Rel2* kd mosquitoes to systemic and oral *Sm* infections

2.2. Examine the contribution of phagocytosis by hemocytes to defense against *Sm* in both models of infection

*Specific Aim 3: Compare the transcriptomes of abdomens and midguts of mosquitoes fed on* Sm *orally to those injected with* Sm

# CHAPTER III

# RESULTS

## A. *Sm* invades the hemolymph after mosquito oral infection

*Sm* efficiently colonizes the gut of *A. gambiae* mosquitoes after oral infection [59, 441]. It also colonizes the ovaries, and is vertically transmitted to the progeny, which renders this bacterium an important tool for paratransgenic approaches that aim to generate mosquitoes resistant to *Plasmodium* infection [59]. Here, we monitored, during three consecutive days, *Sm* dynamics in the gut and hemolymph of mosquitoes feeding continuously on *Sm* DB11 strain suspended in 3% sucrose solution. *Sm* DB11 is known to be virulent to insects, nematodes and mice [440, 481, 482]. The results showed that *Sm* CFUs in the hemolymph are high on day 1, then drop on days 2 and 3 after oral infection despite continuous feeding on *Sm* (Fig. 14a). Even though the highest numbers of *Sm* CFUs were detected on day 1, they were generally low, not exceeding 250 CFUs per mosquito. A similar trend was observed in the gut, whereby *Sm* CFUs dropped significantly by days 2 and 3 after oral infection; however, the guts generally contained much higher numbers (approximately 10000-folds more) of *Sm* CFUs at all three days, relative to the hemolymph (Fig. 14b), suggesting that only few bacteria are present in the hemolymph at a given time. There were no significant differences in *Sm* CFUs in the sugar pads between all three days that could explain the significant drop observed in the gut CFUs at days 2 and 3 (Fig. 14c), indicating that *Sm* remains viable in the sugar solution for several days.

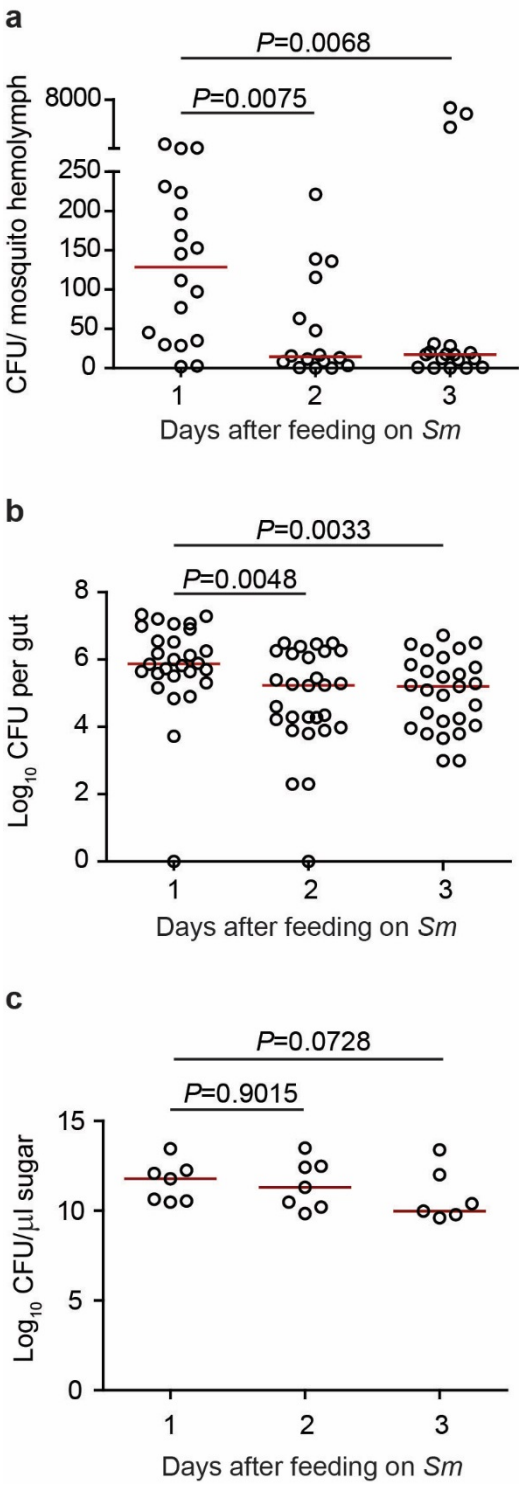


Figure 14. *Sm* acquired through the oral route crosses the midgut epithelium into the hemolymph.

(**a**) Hemolymph was collected by perfusion from batches of 5 mosquitoes each, at the indicated time points post-feeding on *Sm*, and plated on Luria Bertani (LB) agar containing the appropriate antibiotic.

Each point on the scatter plot represents the mean CFU per batch per mosquito. Data were pooled from five independent biological experiments. (**b**) Guts of individual mosquitoes were dissected at the indicated time points post-feeding on *Sm*, homogenized and plated on LB agar containing the appropriate antibiotic. Data were pooled from three independent biological experiments. Each point on the scatter plot represents one midgut. (**c**) Bacterial counts were monitored in sugar pads harboring *Sm* ($OD_{600}$=1) over a period of three days. Data shown are from seven independent biological experiments. Each point on the scatter plot corresponds to CFU/µl of sugar solution in the pad per experiment. Medians are represented by red lines. Statistical analysis was performed using Mann Whitney test, and medians were considered significantly different if $P<0.05$.

## B. The route of hemolymph invasion by *Sm* alters the contribution of CTL4 and TEP1 to bacterial clearance

The fact that *Sm* invades the mosquito hemolymph in low numbers after oral infection, mimicking natural bacterial infections in the field, renders it an attractive model to address whether the route of hemolymph invasion (thoracic injection *versus* crossing of the midgut) influences the contribution of key humoral immune factors to systemic antibacterial defense. To that purpose, we selected CTL4 and TEP1 as candidates due to their essential role in the systemic immune response against septic, i.e., through thoracic injection, Gram-negative bacterial infections [116, 155, 301, 310], specifically CTL4, which forms a heterodimeric complex with the lectin CTLMA2 that protects mosquitoes from septic *E. coli*, *Pseudomonas*, and *Enterobacter cloacae* infections [155]. First, we assessed the contribution of these genes to the susceptibility of *A. gambiae* adult female mosquitoes to septic *Sm* infections established through thoracic injection. Mosquitoes treated with gene-specific double stranded RNA (dsRNA) for CTL4 and TEP1 were injected with a *Sm* suspension in phosphate buffered

saline (PBS) ($OD_{600}$=0.0005) at day 3 after dsRNA administration. At this OD, the CFUs injected per mosquito ranged between 19 and 113. Both, *CTL4* and *TEP1* kd compromised mosquito survival to injected *Sm* (Fig 15a, b; See appendix Fig. 1a, b) compared to *LacZ* kd control. Also, *Sm* proliferation in these genotypes was significantly higher than in the control group (Fig. 15c, d). The *Sm* DB11 strain used herein is gentamycin-resistant and expresses DsRed [440], which allows accurate measurement of CFUs in whole mosquito lysates, without interference from natural *Sm* strains whose presence seems to be sporadic and minor in our mosquitoes (Fig. 16). This is further corroborated by a recent published work from our lab, in which the total operational taxonomic units (OTUs) belonging to the genus *Serratia* in the midguts of *A. gambiae* mosquitoes collected from our insectary over a 7-months period was about 12% [483]. Western blot analysis showed that both TEP1 and CTL4 were efficiently knocked down (Fig. 15e).

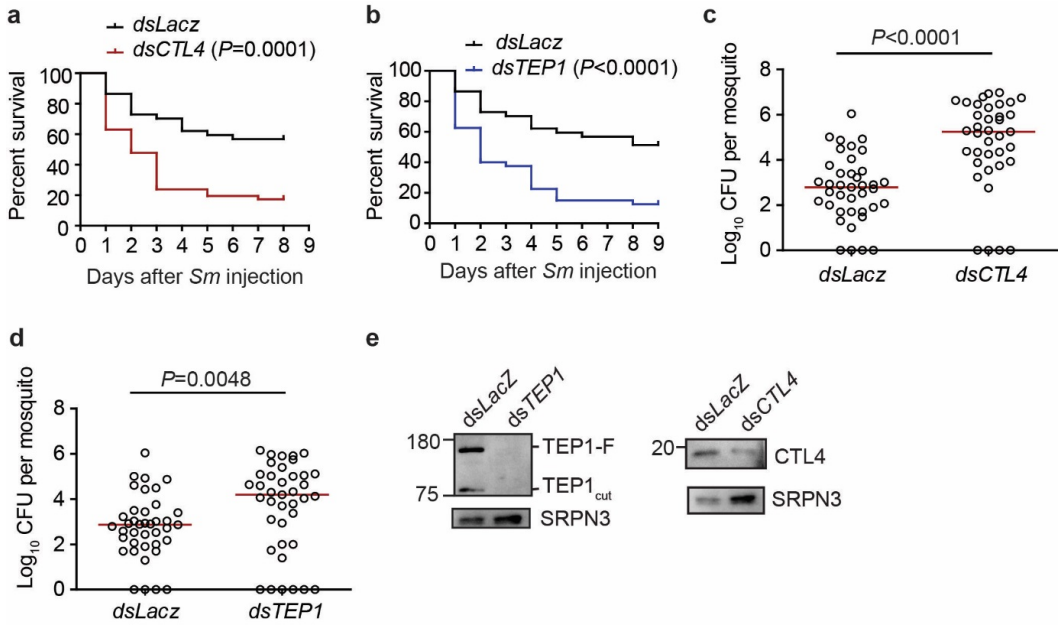


Figure 15. TEP1 and CTL4 are required for defense against systemic *Sm* infections.

**(a, b)** Survival assays of the indicated mosquito genotypes after injection with *Sm* ($OD_{600}$ = 0.0005).One representative experiment is shown from at least three independent biological experiments. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test. (**c**, **d**) Bacterial proliferation assays conducted on the indicated mosquito genotypes injected with *Sm* ($OD_{600}$ = 0.0005). Batches of 8 whole mosquitoes were grinded each in LB medium at 24 h after infection and CFUs were scored on LB plates supplemented with the appropriate antibiotic. Each circle on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using Mann Whitney test, and medians were considered significantly different if $P<0.05$. Data shown are from 9 independent biological experiments. (**e**) Western blots showing the knockdown efficiencies of TEP1 and CTL4. αSRPN3 was used to control for loading.

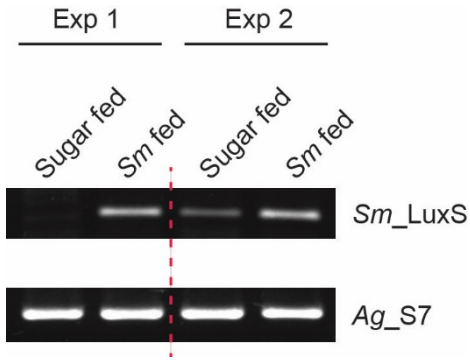


Figure 16. The natural presence of *Sm* in the *A. gambiae* G3 colony is sporadic.

Detection of *Sm* in the mosquito colony using primers specific to *Sm LuxS* gene. For each sample on the gel, DNA was extracted from 10 midguts dissected from either sugar fed mosquitoes (Sugar_fed) or mosquitoes that have been feeding on *Sm* DB11 strain for 24 hours (*Sm*_fed). Shown are two independent experiments separated by dashed red line. Polymerase chain reaction (PCR) amplification of the gene encoding *A. gambiae* ribosomal protein S7 (Ag_S7) was used to control for loading.

Interestingly, when the same strain was used to establish systemic infections through oral feeding in *CTL4* and *TEP1* kd mosquitoes, no effect on survival was

observed relative to the control group (Fig. 17a; See appendix, Fig. 2). We performed hemolymph perfusions at 72 h after oral infection to score the numbers of *Sm* that invaded the hemocoel in the different mosquito genotypes. Our data showed that *Sm* CFUs in the hemolymph of *CTL4* and *TEP1* kd mosquitoes were low and similar to those in the control (Fig. 17b); median values were 84.8, 43.6, and 42.2 for *LacZ*, *CTL4* and *TEP1* kd mosquitoes, respectively. This indicates that the immune function of these proteins becomes non-essential when *Sm* invades the hemolymph through the oral route. The differential contribution of CTL4 and TEP1 to immune defense against *Sm* in the two routes of infection could be attributed to differences in the numbers of *Sm* introduced into the hemolymph between both routes; 19 to 113 CFUs of *Sm* were injected into the hemolymph during septic infections which proliferated to reach around 616 (Fig.15c) and 733 (Fig.15d) CFUs in *LacZ* kd mosquitoes, while the numbers of *Sm* that reached the hemolymph of wildtype mosquitoes at 24 h after oral infection ranged from 2 to 280 CFUs (Fig. 14a). However, the fact that *Sm* proliferated dramatically in *CTL4* and *TEP1* kd mosquitoes at 24 h after *Sm* injection (Fig. 15c, d), while in oral infections *Sm* CFUs in the hemolymph remained low in these genotypes, even at 72 h after feeding (Fig. 17b) argues otherwise.

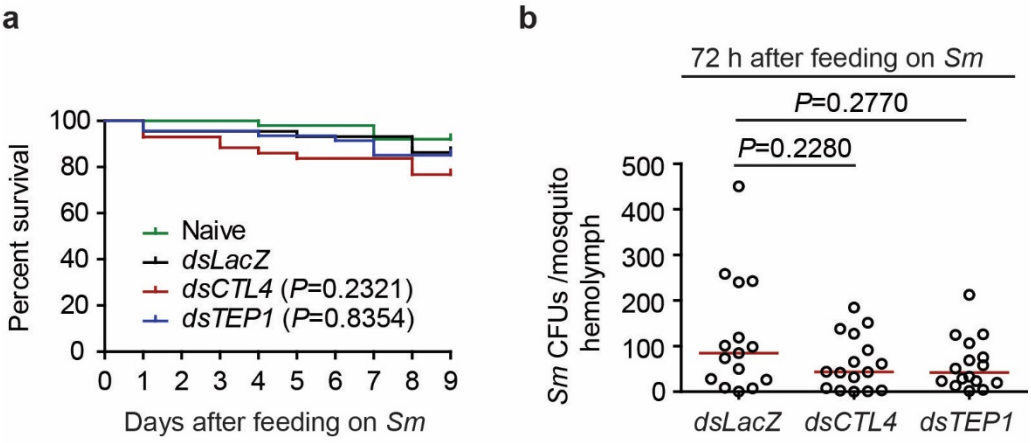

Figure 17. CTL4 and Tep1 are not required for defense against *Sm* oral infections.

(**a**) Survival assays of the indicated mosquito genotypes after oral infection with *Sm* ($OD_{600}$ = 1). One representative experiment is shown from at least three independent biological experiments. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test. (**b**) Bacterial proliferation assays conducted on the indicated mosquito genotypes after oral infection with *Sm* ($OD_{600}$ = 1). Hemolymph was perfused from batches of five mosquitoes 72 h post-feeding on *Sm* and CFUs were scored on LB plates supplemented with the appropriate antibiotic. Each circle on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test. Medians (black lines) were considered significantly different if $P<0.05$. Data shown are from three independent biological experiments.

To try to explain the controlled proliferation of *Sm* in the hemolymph after oral feeding, we hypothesized that the process of midgut invasion might influence the fitness of bacterial cells possibly due to exposure to oxidants (reactive oxygen and nitrogen species) generated as part of the local epithelial immune response, and which are known to damage bacterial cells (reviewed in [484]). For instance, oxidants generated by dual oxidase in the *Drosophila* gut limit microbial proliferation [445], and nitric oxide produced in the *A. gambiae* midgut by heme peroxidase 2 and NADPH oxidase 5 enhance *Plasmodium* cytotoxicity [277]. To determine if bacteria exhibit an altered fitness after crossing the midgut, hemolymph was collected by perfusion from wildtype mosquitoes that have fed on *Sm* during 24 h, and bacterial cells in the perfusate were pelleted by centrifugation, washed and injected into *LacZ* and *CTL4* kd naïve mosquitoes. The same mosquito genotypes injected with *Sm* ($OD_{600}$=0.0005) prepared from a fresh batch culture were used as control. Challenged mosquitoes were

homogenized 24 h later to score *Sm* CFUs. The results show that bacteria prepared from hemolymph perfusates were able to proliferate to the same extent as those originating from a fresh culture, indicating that bacterial cells that cross the gut into the hemolymph do not seem to suffer from a reduced growth fitness (Fig. 18).

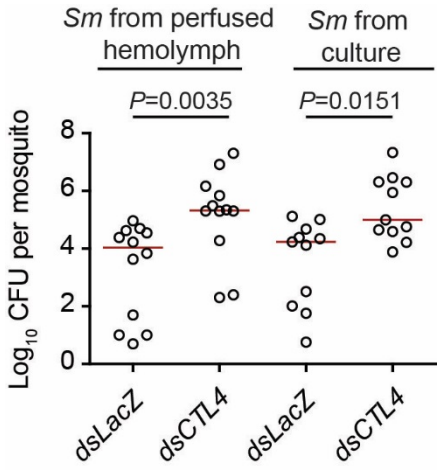


Figure 18. Invasion of the midgut epithelium does not alter the fitness of *Sm*.

Bacterial proliferation assays conducted on the indicated mosquito genotypes after injection with *Sm* prepared from a fresh bacterial culture ($OD_{600}$ = 0.0005) or collected from hemolymph perfusions of mosquitoes that have fed on *Sm* for 24 h. Batches of 7 whole mosquitoes were grinded in LB medium at 24 h after infection and CFUs were scored 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 significantly different if $P<0.05$. Data shown are from three independent biological experiments.

## C. The Rel2 pathway and phagocytosis are dispensable for defense against *Sm* systemic infections established after gut invasion

The mosquito Rel2 pathway is involved in defense against systemic infections with

Gram-negative and Gram-positive bacteria [68, 230], but also protects against *P. falciparum* ookinetes [68, 184, 299]. To determine the contribution of Rel2 to systemic defense against *Sm* that invade the hemolymph following an oral infection, *LacZ* (control), *Rel2* and *Rel1* kd mosquitoes were fed continuously on sugar pads containing a suspension of *Sm* at an $OD_{600}$=1, and their survival scored over 10 days post-challenge. Neither Rel1 nor Rel2 silencing compromised mosquito survival to oral *Sm* infections (Fig. 19a; See appendix, Fig. 3a). In contrast, when systemic infection in these mosquito genotypes was established by injecting *Sm* into the hemocoel, *Rel2* kd compromised mosquito survival (Fig. 19b; See appendix, Fig. 3b) and resistance (Fig. 19c) as noted from the enhanced bacterial proliferation relative to control. Hence, the Rel2 pathway contributes to immune defense against *Sm* systemic infections established through septic injury but not through feeding. The efficiency of Rel1 and Rel2 silencing in our hands is 50% and 44% (Fig. 19d), which is similar to what was reported previously for these genes [230, 231].

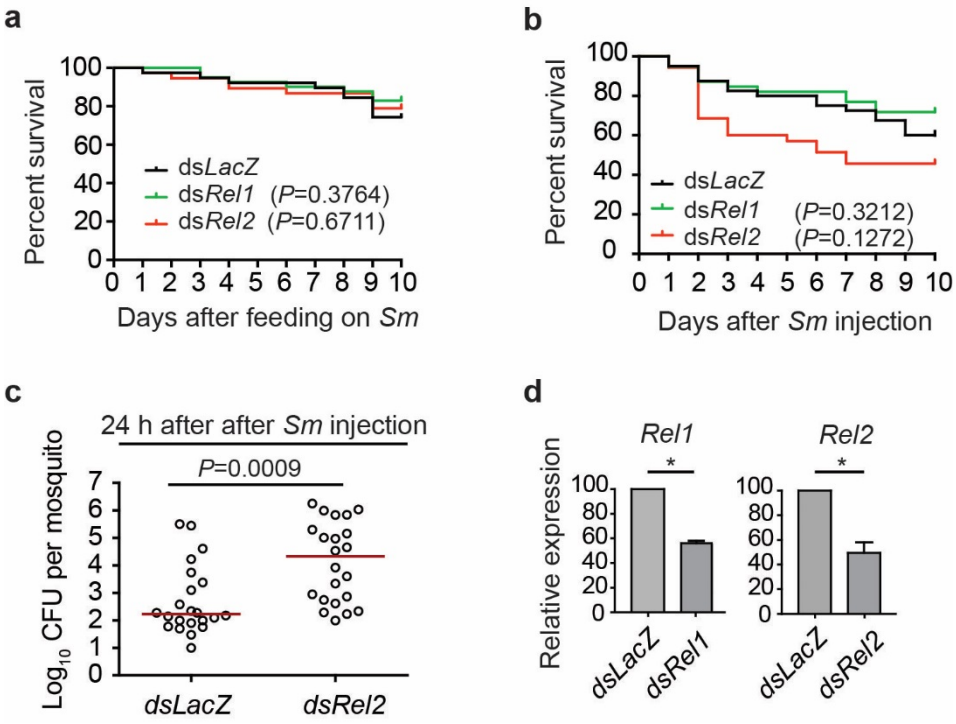


Figure 19. The Rel2 signaling pathway plays a role in mosquito tolerance and resistance against systemic but not oral *Sm* infections.

(**a**, **b**) Survival assays following *Sm* oral ($OD_{600}$=1) (**a**) and systemic ($OD_{600}$=0.0005) (**b**) infections in mosquitoes silenced for either *Rel1* or *Rel2*. One representative experiment is shown from at least three independent biological experiments. The Kaplan-Meier survival test was used to calculate percent survival. Statistical significance of the observed differences was calculated using the log-rank test. (**c**) Bacterial proliferation assays conducted on *Rel2* kd mosquitoes injected with *Sm* ($OD_{600}$ = 0.0005). Batches of 8 whole mosquitoes were grinded in LB medium at 24 h after infection and CFUs were scored 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. Data shown are from at least three independent biological experiments. (**d**) Transcript levels of Rel1 and Rel2 measured by quantitative real time (qRT)-PCR in whole female mosquitoes at four days following injection of their respective dsRNA. Error bars represent standard error of the mean of two biological repeats. Statistical analysis was done using the student's t-test.

Phagocytosis is an important innate immune response that was shown to control host susceptibility to septic bacterial infections in *A. gambiae* [310, 405] and *Drosophila* [485-488]. Additionally, blocking phagocytosis in *Drosophila* adults by cytochalasin D injection compromised the survival of the flies to oral *Sm* infections [440]. Based on these data, we hypothesized that the dispensable roles of CTL4, TEP1 and the Rel2 pathway in defense against *Sm* that gain access into the hemolymph after oral infection, may be due to a primary role of phagocytosis in controlling host susceptibility through this route. To address this point, mosquitoes injected intrathoracically with 69 nl of a 62.5 μg/ml solution (120 μM) of cytochalasin D in PBS were allowed to feed continuously on a sugar solution containing *Sm* at 6 hours after cytochalasin D injection. Survival assays revealed that cytochalasin D treatment did not affect mosquito susceptibility to oral *Sm* infections (Fig. 20a; See appendix, Fig. 4a), despite the fact that the concentration of cytochalasin D used herein is higher than that which blocked phagocytosis in *Drosophila* adults [440] and *A. gambiae* cell lines [404]. Hence, our data suggest that, in the mosquito, phagocytosis may not play an essential role in controlling *Sm* that escape into the hemolymph after oral infections. Also, cytochalasin D treatment did not compromise mosquito survival to *Sm* injection (Fig. 20b; See appendix, Fig. 4b), which was not surprising to us due to the primary immune defensive role of the humoral factors TEP1 and CTL4 in this infection route.

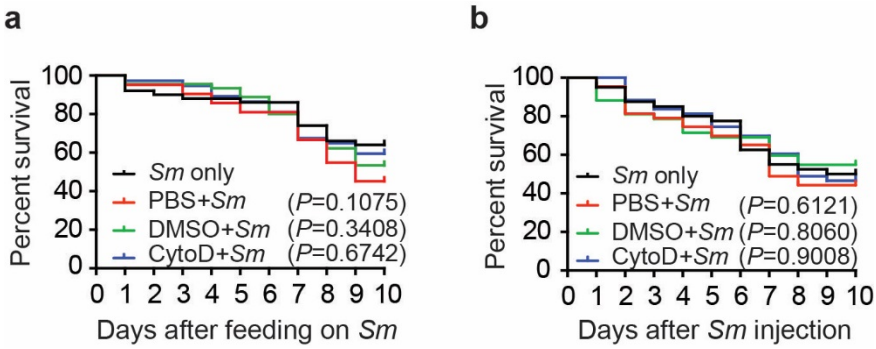


Figure 20. Blocking phagocytosis by cytochalasin-D doesn't seem to significantly impact mosquito susceptibility to oral or systemic *Sm* injections.

Survival of non-injected mosquitoes or mosquitoes pre-injected with either of PBS, DMSO, or cytochalasin-D was monitored over a period of 10 days following (**a**) oral ($OD_{600}$ = 1) or (**b**) systemic *Sm* infections. One representative experiment is shown from at least three independent biological experiments. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

## D. Abdomen and midgut transcriptional responses after *Sm* oral and septic infections

The fact that CTL4 and TEP1-mediated systemic immune responses did not provide resistance to *Sm* invading the hemolymph from the gut in addition to the dispensable roles of the Imd pathway and phagocytosis in this route prompted us to monitor whether oral infection primes the tissue-specific expression of an immune gene repertoire in the fat body or midgut that could explain the dispensable roles of CTL4 and TEP1 in this route of infection. To that purpose, abdomens (excluding gut, Malpighian tubules and ovaries) and midguts (excluding hind- and foreguts) were dissected from wildtype mosquitoes at 6, 12 and 24 h after feeding on a 3% sucrose solution containing *Sm*

Db11 ($OD_{600}$=1) or after injection with a *Sm* suspension in PBS ($OD_{600}$=0.0005), and transcriptional responses were monitored by RNA-seq. Mosquitoes fed on 3% sucrose solution or injected with sterile PBS were used as controls for *Sm* oral infection and *Sm* injection, respectively. Three independent biological experiments were performed. All differentially expressed transcripts (DETs) were determined according to a false discovery rate of 0.05 (See appendix, Table 1). In the abdomens, 70, 87 and 123 DETs were identified, respectively, at 6, 12 and 24 h after *Sm* oral infection with respect to untreated controls, i.e., only sugar fed, whereas, 16, 11 and 47 DETs were identified at the respective time points after *Sm* injection with respect to PBS injection only (Fig. 21a, c), indicating that oral infection triggers more profound transcriptional changes in the abdomen than injection. In midguts, 406, 296 and 106 DETs were identified at 6, 12 and 24 h after *Sm* oral infection with respect to untreated controls, whereas, 16, 12 and 12 DETs were identified at the respective time points after *Sm* injection (Fig. 21b, d), again indicating that oral infection had a greater influence on the midgut transcriptome than injection, which is rather expected since in the context of direct injection into the hemocoel, *Sm* is unlikely to invade the gut epithelium from the basal side. The low numbers of DETs in abdomens and midguts of *Sm* injected mosquitoes are also likely due to the fact that PBS injection itself regulated a large number of transcripts in these tissues at all three time points (Fig. 21e, f). Of note, although the PBS solution used in these treatments is sterile, bacteria attached to the mosquito cuticle can still be introduced into the hemolymph due to the wounding process.

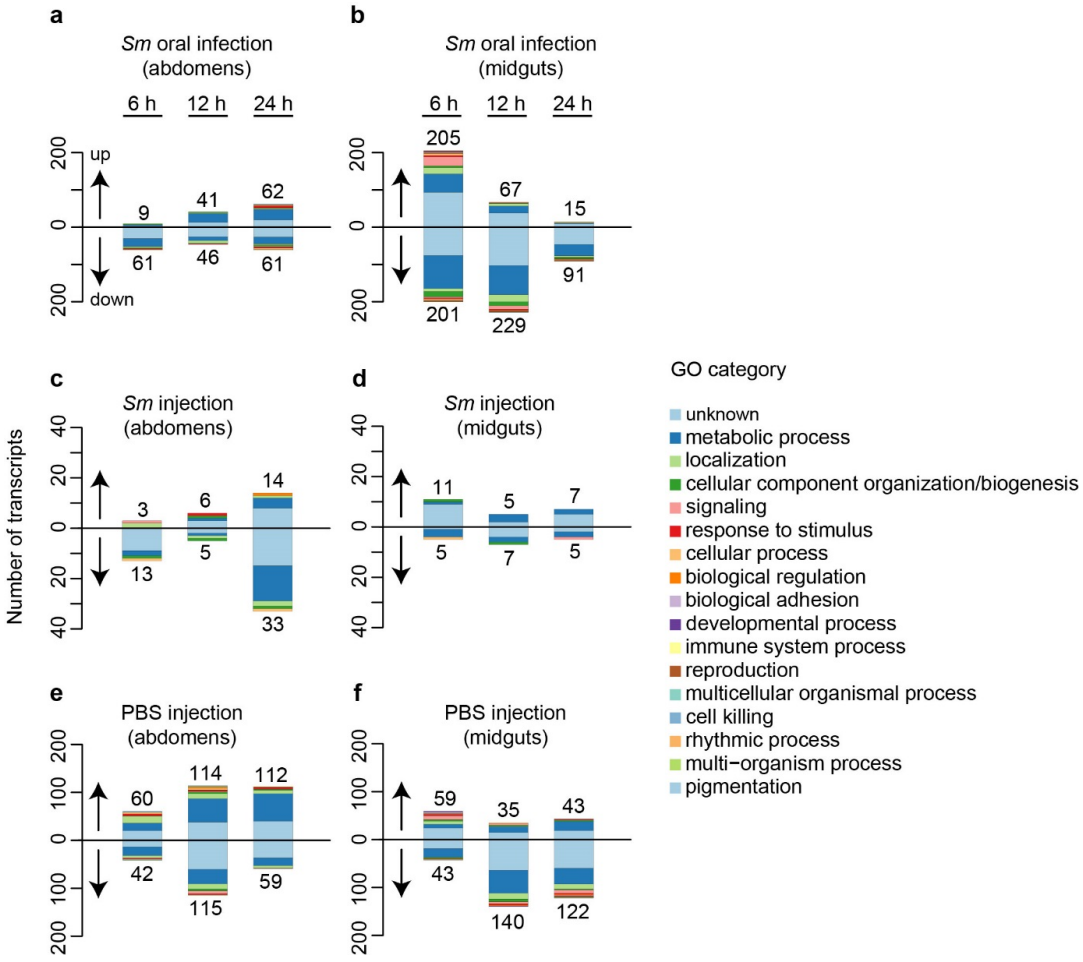


Figure 21. Number of differentially expressed transcripts per treatment.

The plot is colored according to top-level gene ontology terms within the "biological process" ontology and each transcript was assigned to its top-level gene ontology term within the "biological process" ontology. Whenever a transcript was associated with more than one top-level term, the least common term was chosen.

Functional classification of all DETs in abdomens and midguts from all treatments revealed that metabolic genes are the most represented class followed by those involved in cellular localization (Fig. 21; See appendix, Table 1). Surprisingly, immunity genes were under-represented in both abdomens and midguts from all treatments suggesting that oral and septic infections with *Sm* have little effect on the transcriptome of

immunity genes. When comparing the DETs in midguts of mosquitoes injected with *Sm* to those of mosquitoes fed on *Sm*, a small overlap was observed (Fig. 22a). The same was noted for abdomens (Fig. 22b), indicating that different physiological responses are triggered in response to the different routes of *Sm* infection.

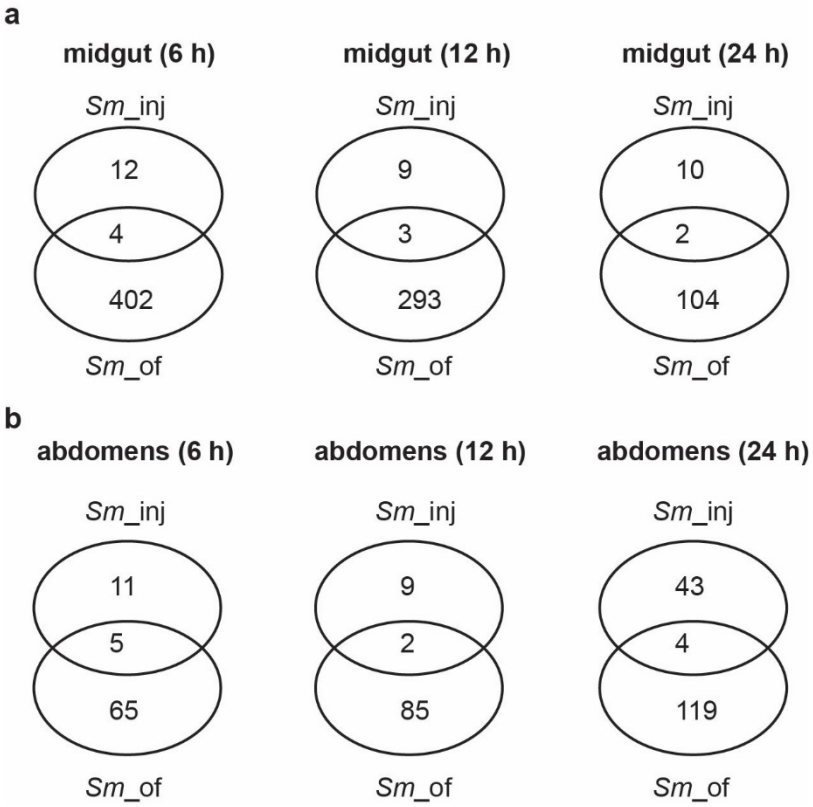


Figure 22. Number of differentially expressed genes in (a) midguts and (b) abdomens of mosquitoes that were fed on *Sm* (*Sm*_of) or injected with *Sm* (*Sm*_inj), at the indicated time points after *Sm* challenge.

The gene expression at the three different time points after *Sm* oral feeding treatment was compared to untreated mosquitoes. The gene expression at the three different time points after *Sm* injection was compared to the gene expression at the corresponding time points after sterile PBS injection. h, hours.

To determine whether hemolymph infection following *Sm* injection or midgut crossing triggers route-specific unique responses to *Sm* in abdomens, we identified the

transcripts whose expression changes significantly in response to *Sm* injection (*Sm*_inj) relative to PBS injected (PBS_inj) control but not in response to any other treatment, i.e., *Sm* oral feeding (*Sm*_of) vs untreated control (UC) or *Sm*_inj vs UC or PBS vs UC, and transcripts whose expression changes significantly in response to *Sm*_of relative to UC but not in response to any other treatment, i.e., *Sm*_inj vs PBS_inj or *Sm*_inj vs UC or PBS_inj vs UC. In abdomens, where physiological responses are expected to be more relevant to hemolymph infection with *Sm* due to the presence of the fat body and sessile hemocytes, only one transcript, vacuolar protein sorting 60 (Vps 60; AGAP005100), showed significant change in expression unique to *Sm*_inj *vs* PBS_inj (Table 1). Vps proteins are involved in the formation of multivesicular bodies which play important roles in the endocytic degradation of proteins, but also in the formation of exosomes [489], which are small extracellular vesicles that mediate intercellular communication to regulate several biological processes including tissue repair [490]. The upregulation of Vps60 in abdomens may reflect enhanced investment in repair processes in response to *Sm* infection. Tissue repair and regeneration processes are crucial for host tolerance to infection [491]. Alternatively, this upregulation may indicate an increase in the protein secretory capacity of the fat body in response to immune activation and infection, which in *Drosophila* was associated with enhanced tolerance to infection [492]. On the other hand, three transcripts showed a down-regulated expression profile in abdomens unique to *Sm*_of vs UC (Table 2) including, CNOT transcription subunit complex 3 (AGAP009030), very long chain enoyl-reductase (AGAP010714) and UPF0518 (AGAP011705). CNOT is a large multi-subunit RNA deadenylase, composed of catalytic and non-catalytic subunits, that is conserved in eukaryotes and plays key roles in mRNA degradation and turnover, hence controlling the rate of protein expression

[493]. It also plays an effector role in miRNA-mediated gene silencing [494]. As such, CNOT is involved in regulating several physiological processes in the cell including, cell death, autophagy, immunity, inflammation, and differentiation to mention a few [493, 495]. It is tempting to speculate that the down-regulation of CNOT3, a non-catalytic subunit essential for CNOT activity [496], in abdomens may increase the stability of mRNAs involved in immunity, tissue repair or stress response which might favor host tolerance to systemic infection established after feeding on *Sm*. The very long chain enoyl-reductase is involved in the synthesis of sphingolipids and glycerophospholipids [497], and its downregulation may indicate a shift in lipid metabolism, whereas UPF0518 has no known function.

Table 1. Transcripts that show a differentially expressed profile unique to midguts and abdomens of mosquitoes injected with *Sm*.

| | Transcript | Treat Up | Treat Down | Gene | Gene Symbol | Description |
|---|---|---|---|---|---|---|
| AGAP029633-RA | AGAP029633-RA | gut_6h | | AGAP029633 | Hex-A | Hexokinase |
| AGAP005942-RB | AGAP005942-RB | | gut_6h | AGAP005942 | | unknown |
| AGAP010387-RB | AGAP010387-RB | | gut_12h | AGAP010387 | | alanine-glyoxylate aminotransferase |
| AGAP005100-RA | AGAP005100-RA | abdomen_24h | | AGAP005100 | Vps60 | vacuolar protein sorting 60 |

GO enrichment analysis of the route-unique transcripts listed in Tables 1 and 2 suggests that the global physiological response associated with the oral route includes most of that associated with *Sm* injection (except 4 genes), in addition to other specific functions (See appendix, Tables 2 and 3). These oral route-specific responses are mainly attributed to the midgut (33 out of 36 genes), and are enriched in biological

processes related mainly to protein translation, protein folding, protein modification, DNA damage repair, cell cycle regulation among others (See appendix, Table 2). These responses could reflect the pathology induced by *Sm* to the midgut epithelium.

Table 2. Transcripts that show a differentially expressed profile unique to midguts and abdomens of mosquitoes fed on *Sm*.

| | Transcript | Treat Up | Treat Down | Gene | Gene Symbol | Description |
|---|---|---|---|---|---|---|
| AGAP000519-RA | AGAP000519-RA | gut_6h | | AGAP000519 | | diacylglycerol kinase (ATP dependent) |
| AGAP000704-RA | AGAP000704-RA | gut_6h | | AGAP000704 | | cell cycle control protein 50A |
| AGAP001859-RA | AGAP001859-RA | gut_6h | | AGAP001859 | | DnaJ homolog subfamily B member 14 |
| AGAP003525-RA | AGAP003525-RA | gut_6h | | AGAP003525 | | CCR4-NOT transcription complex, subunit 2 |
| AGAP003595-RA | AGAP003595-RA | gut_6h | | AGAP003595 | | rRNA biogenesis protein RRP5 |
| AGAP006101-RA | AGAP006101-RA | gut_6h | | AGAP006101 | | unknown |
| AGAP006681-RA | AGAP006681-RA | gut_6h | | AGAP006681 | | unknown |
| AGAP008822-RA | AGAP008822-RA | gut_6h | | AGAP008822 | | FK506-binding protein 14 |
| AGAP009030-RA | AGAP009030-RA | gut_6h | abdomen_24h | AGAP009030 | | CCR4-NOT transcription complex subunit 3 |
| AGAP009290-RA | AGAP009290-RA | gut_6h | | AGAP009290 | | cohesin loading factor subunit SCC2 |
| AGAP011166-RA | AGAP011166-RA | gut_6h | | AGAP011166 | | protein phosphatase 1, catalytic subunit |
| AGAP011701-RA | AGAP011701-RA | gut_6h | | AGAP011701 | | predicted G-protein coupled |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | receptor GPCR |
| AGAP000308-RA | AGAP000308-RA | | gut_6h | AGAP000308 | | proteasome activator subunit 3 (PA28 gamma) |
| AGAP000852-RA | AGAP000852-RA | | gut_6h | AGAP000852 | | Small ubiquitin-related modifier |
| AGAP001499-RB | AGAP001499-RB | | gut_6h | AGAP001499 | GPRMTN | putative melatonin receptor |
| AGAP001545-RA | AGAP001545-RA | | gut_6h | AGAP001545 | | ribosomal biogenesis protein RLP24 |
| AGAP003517-RB | AGAP003517-RB | | gut_6h | AGAP003517 | | phenylalanyl-tRNA synthetase beta chain |
| AGAP004064-RB | AGAP004064-RB | | gut_6h | AGAP004064 | | RNA-binding protein Nob1 |
| AGAP004151-RA | AGAP004151-RA | | gut_6h | AGAP004151 | | protein phosphatase 5 |
| AGAP004238-RA | AGAP004238-RA | | gut_6h | AGAP004238 | CCT1 | Chaperonin containing TCP1 subunit 1 |
| AGAP004481-RA | AGAP004481-RA | | gut_6h | AGAP004481 | mRpS26 | 28S ribosomal protein S26, mitochondrial |
| AGAP004632-RA | AGAP004632-RA | | gut_6h | AGAP004632 | DEF2 | Defensin 2 |
| AGAP005122-RA | AGAP005122-RA | | gut_6h | AGAP005122 | | UBX domain-containing protein 1 |
| AGAP006130-RA | AGAP006130-RA | | gut_6h | AGAP006130 | eIF3l | Eukaryotic translation initiation factor 3 subunit L |
| AGAP006944-RA | AGAP006944-RA | | gut_6h | AGAP006944 | eIF3e | Eukaryotic translation initiation factor 3 subunit E |
| AGAP007668-RA | AGAP007668-RA | | gut_6h | AGAP007668 | eIF3g | Eukaryotic translation initiation |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | factor 3 subunit G |
| AGAP010572-RA | AGAP010572-RA | | gut_6h | AGAP010572 | | protein LTV1 |
| AGAP011644-RA | AGAP011644-RA | | gut_6h | AGAP011644 | | inositol-1,4,5-trisphosphate 5-phosphatase |
| AGAP011922-RA | AGAP011922-RA | | gut_6h | AGAP011922 | | Lipase maturation factor |
| AGAP012590-RA | AGAP012590-RA | | gut_6h | AGAP012590 | | MutT domain protein-like protein |
| AGAP000767-RA | AGAP000767-RA | gut_12h | | AGAP000767 | | membrane-associated progesterone receptor component 2 |
| AGAP006030-RA | AGAP006030-RA | gut_12h | | AGAP006030 | mfrn | Mitoferrin |
| AGAP006652-RB | AGAP006652-RB | | gut_24h | AGAP006652 | | ubiquitin carboxyl-terminal hydrolase 10 |
| AGAP008988-RA | AGAP008988-RA | | gut_24h | AGAP008988 | | Glutamine synthetase |
| AGAP010714-RA | AGAP010714-RA | | abdomen_6h | AGAP010714 | | very-long-chain enoyl-CoA reductase |
| AGAP011705-RA | AGAP011705-RA | | abdomen_24h | AGAP011705 | | UPF0518 protein |

A Wallenius non-central hypergeometric distribution was used to test for the enrichment of GO terms, KEGG pathways and gene families in the total set of differentially regulated genes in abdomens and midguts of mosquitoes fed on or injected with *Sm*, relative to untreated (UC) and PBS injected controls, respectively. The results identified 13 unique terms (4 gene families, 5 KEGG pathways and 4 GO terms) that were significantly over-represented, the majority of which were associated with functions related to protein translation, processing and export, followed by terms related to metabolic processes, in particular oxidative phosphorylation, and one associated with immunity (See appendix, Table 4). Concerning immunity, only the CLIP family was

significantly over-represented in the midgut of mosquitoes at 12 h after *Sm* oral infection (Fig. 23; See appendix, Table 4). In total, 10 CLIPs were downregulated in this treatment including CLIPC4, CLIPB4, CLIPB1, CLIPC9, CLIPB13, CLIPA8, CLIPA4, CLIPA6, CLIPA1 and CLIPA7. CLIPs are key components of serine protease cascades that regulate important insect immune responses specifically melanization and Toll pathway activation [350, 471, 498]. Of note, the KEGG pathway enrichment analysis identified 12 genes involved in oxidative phosphorylation that are all downregulated in the midgut after feeding on *Sm*, suggesting that midgut infection may be triggering a shift in the gut metabolic program. As for the abdomens, only the forkhead transcription factors (FOXO) signaling pathway is enriched after *Sm* injections but not feeding (See appendix, Table 4). In *Drosophila*, FOXO transcription factor activates AMP production under nutritional stress independent of Toll and Imd pathways [499]. FOXO is also required for *Drosophila* to survive oral infections with *Sm* [500]. Whether FOXO signaling plays a similar role in mosquito immunity against bacterial septic injections and oral infections remains to be elucidated.

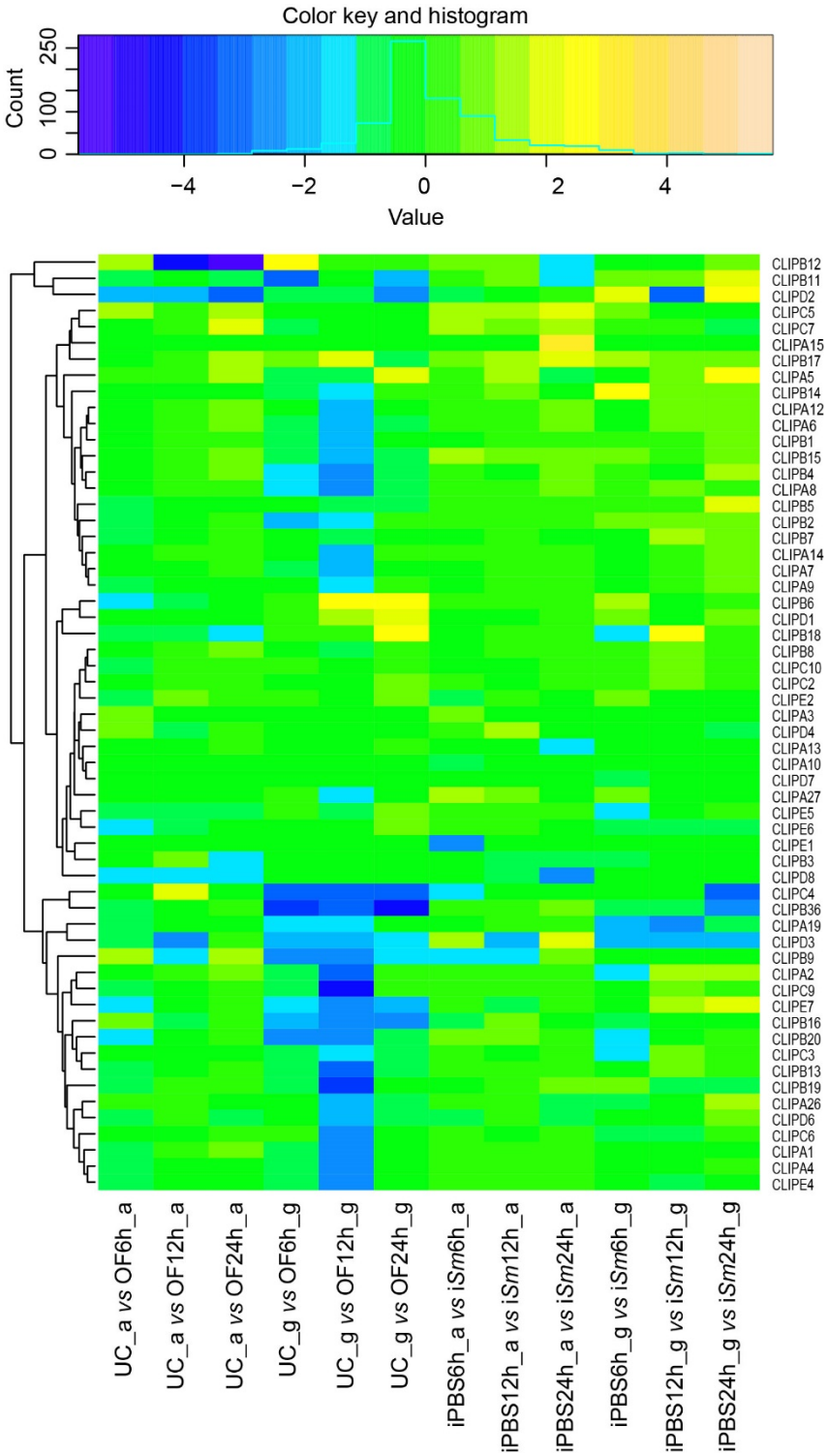


Figure 23. Heatmap of estimated log-fold changes of all CLIP genes.

Log-fold changes were estimated for each comparison indicated by column labels. The column labels start with symbol for control (UC = unchallenged, iPBS = PBS injection), followed by a symbol for the

tissue (a = abdomen, g = gut). The symbols for the treatments are OF = oral feeding, i*Sm* = injection of *Serratia marcescens*. The timing of treatment is indicated in hours (h). The row dendrogram shows a hierarchical clustering of the dissimilarities between CLIP genes in their log-fold change patterns among all comparisons shown in the plot.

In addition to CLIPs, few other genes belonging to distinct immune gene families, though not over-represented, were also downregulated after *Sm* oral but not septic infections (See appendix, Table 1). These include, Eiger, GNBPB1, the scavenger receptors SCRB5, SCRB7 and SCRB9, PPO6, TEP2, and CTLMA1. Eiger was downregulated in abdomens at 24 h after *Sm* oral infections. It is a TNF orthologue which, in *Drosophila*, is also expressed in the fat body [501] and plays an important role in regulating melanization, antimicrobial peptide expression and immune defense against extracellular pathogens [501, 502]. GNBPB1 was also downregulated in abdomens at 24 h after *Sm* oral infections, and it was previously shown to contribute to anti-*Plasmodium* immunity [133]. Two members of the scavenger receptor gene family, SCRB5 and SCRB7 were downregulated in abdomens at 12 and 6 h after *Sm* oral infections, respectively, while SCRB9 was downregulated in the midgut at 6 h after infection. The role of these receptors in mosquito immunity has not been investigated, but members of this family are involved in the phagocytic uptake of bacteria in *Drosophila* [503-505]. PPO6, TEP2 and CTLMA1 were also downregulated in the midgut in response to *Sm* oral infection. PPO6, a phenoloxidase expressed in mosquito adults, is involved in the melanization reaction to *P. berghei* ookinetes, bacteria and fungi [278, 311, 386]. While the roles of TEP2 and CTLMA1 in immune defense are unknown, certain members of the TEP and CTL families are key players in the mosquito anti-microbial defense [116, 155, 156, 159, 278, 295]. On the other hand, only

four immunity genes were upregulated after *Sm* oral infections; Galectin 5 and CecA were upregulated in abdomens, whereas, C-type lectin 6 (CTL6) and lysozyme C7 (LYSC7) were upregulated in the midgut at the indicated time points (See appendix, Table 1). Whether these genes are involved in controlling *Sm* proliferation in the hemolymph following oral infection will require further investigations. Altogether, these results suggest that *Sm* invasion of the hemolymph following oral infection is seemingly associated with transcriptional suppression of several immune genes involved in different facets of the humoral and cellular immune response.

# CHAPTER IV

# DISCUSSION

In all organisms, the vast majority of microbial infections are established through initial interactions between microbes and host at barrier epithelia. There is growing evidence, in both invertebrates and vertebrates, that the route of infection determines the adaptive strategies of the host in terms of the nature of immune responses engaged to deal with the insult [506, 507]. In *Drosophila*, oral and systemic infections with *Pseudomonas entomophila* triggered the evolution of resistance in fly populations that was infection-route specific [506]. Also, oral infection of *Drosophila* with different RNA viruses revealed different patterns of virus clearance and immune priming compared to systemic injections [507]. In a similar context, *Anopheles coluzzii* oral infections with O'nyong nyong arbovirus shared little overlap in transcriptional responses with intrathoracic injections [508]. Route-specific immune responses have been also described in mammals. For instance, the intranasal administration of vaccinia virus to mice triggered a stronger adaptive response in magnitude and diversity compared to local intradermal injections [509]. In another study, infection of mice with *Brucella melitensis* through three different routes, intradermally, intraperitoneally and intranasally, revealed route-specific contributions of the three lymphoid populations, Cluster Difference 4 $(CD4)^+$ T cell, B cells and $\gamma\delta^+$ T cells [510]. Interestingly, the authors also showed that the type IV secretion system which is required for *Brucella* persistence in the lungs after intranasal infections, does not seem to promote persistence in the skin after intradermal infections, suggesting that the route of infection not only

influences the physiology of the immune response but also the contribution of certain virulence factors to microbial persistence. However, how the route of infection shapes host-pathogen interaction in *A. gambiae* mosquitoes has not received much attention at the experimental level. In this work, we used *Sm* DB11 strain as a model mosquito pathogen to score the contribution of two key humoral anti-bacterial factors, TEP1 and CTL4, to immune defense against hemolymph infections established either through *Sm* injection or midgut invasion after *Sm* oral feeding, in adult *A. gambiae* female mosquitoes, and to try to identify mosquito physiological responses that are specific to *Sm* infection route.

### A. *Sm* dynamics: The escape from the intestinal tract

*Sm* is detected in the mosquito hemolymph one day after oral infection, a clear indication that *Sm* is able to cross the midgut epithelial barrier and gain access to the body cavity, but the numbers drop significantly during the following two days concomitant with a reduction in *Sm* CFUs in the midguts. This reduction is not due to increased bacterial death in the sugar pads used to feed mosquitoes, since bacterial CFUs in the pads did not change significantly during this period. The drop in *Sm* levels in midguts may reflect a reduction in mosquito feeding due to chronic infection of the gut by *Sm*. In fact, the *Gr9* was shown to be associated with *Sm* infection phenotype of *A. gambiae* midguts, and silencing this gene increased *Sm* colonization of the midgut, indicating that *Sm* infection may trigger a behavioral feeding response [441]. The composition of the gut microbiota in *Drosophila* also influences its foraging behavior [511]. This drop in *Sm* numbers in the hemolymph at days 2 and 3 after feeding on *Sm* may be also attributed to immune defenses active at the level of the midgut epithelium

that may restrict the numbers of bacteria that successfully invade the midgut into the hemolymph [440, 441] or to immune defenses that take place in the hemolymph after successful passage through the midgut barrier, i.e., phagocytosis [440]. We did not address whether blood feeding would influence the dynamics of *Sm* invasion of the hemolymph, however, we expect that it would be more difficult for *Sm* to cross the midgut during blood feeding, since the peritrophic matrix [444] and the dityrosine network produced by a peroxidase dual oxidase system [330], will likely restrict midgut permeability to microbes. Another aspect that we didn't address is whether the actual passage of invading *Sm* occurs in between epithelial cells or rather involves an intracellular route. Indeed, Nehme *et al.* provided evidence of an inherent ability of ingested *Sm* to cross intact epithelia through the cells. Interestingly, *Sm* was always detected within a vacuole [440]. Presumably, intercellular invasion would take place at later times of the infection, given that the integrity of the midgut becomes affected.

### B. Tep1 and CTL4 are dispensable for defense against oral *Sm* infections

We found that CTL4 and TEP1 are required for mosquito resistance to *Sm* infections of the hemolymph established following injection but not oral infection. One possible explanation for the dispensable roles of CTL4 and TEP1 in resistance to *Sm* in the latter route could be that the oral route triggers systemic immune priming by gut epithelia creating redundancy in bacterial defense among different arms of the immune response. Another possible explanation is that perhaps *Sm* doesn't express the same virulence program in both routes of infection, possibly as a result of its exposition to midgut defenses. In fact, it is known that exposure to the midgut triggers some alterations of the outer membrane of bacteria, resulting in changes in susceptibility to immune effectors [512].

## C. Are invading *Sm* proliferating in the hemolymph?

We observed that bacteria invading the hemolymph from the gut remained at low numbers, as compared to those injected directly into the hemolymph, even in control (ds*LacZ*) mosquitoes. We can infer from the hemolymph perfusions assays that an average of 100 bacteria are present in the hemolymph at 24 hrs post feeding on *Sm*, but it remains unclear whether the bacteria that have traversed the epithelium are proliferating in the hemolymph. One possibility is that invading *Sm* retain their proliferative capacity and the increase in bacterial counts is counterbalanced by immune clearance. Another possibility is that a longer generation time, i.e., reduced cell division rate, is associated with bacteria that cross the midgut into the hemolymph but not with those directly injected into the hemolymph. Reduced proliferation is expected to benefit bacterial persistence, since the release of cell wall components, such as peptidoglycan, during bacterial cell division would activate PGRPs leading to Imd pathway activation in fat body cells [115, 124, 513, 514]. However, it is worth noting that the mosquito complement-like system, which plays a key role in anti-bacterial immunity, may not be sensitive to bacterial proliferation since TEP1 was shown to efficiently bind *E. coli* bioparticles [300, 301]. We showed that bacteria that invade the hemolymph from the midgut proliferate efficiently after extraction and injection into the hemolymph of control or ds*CTL4* mosquitoes, indicating that they have not lost fitness. These results suggest that the combination of the midgut invasion process and the exposure to the hostile hemolymph environment may impose a certain stress on the bacteria associated with a reduced proliferation rate. This stress may have been relieved through the process of extraction and washing before the cells are injected into another mosquito.

## D. Melanization doesn't seem to play an essential role in the oral route

Our attempts to measure PO activity following oral *Sm* infections were not conclusive, as some trials showed activation will others did not (data not shown). There are two plausible explanations for this inconsistency: First, hemolymph invasion after oral infection is likely to occur in waves and not at one single time point which makes it difficult to pinpoint the optimal time point for measuring hemolymph PO activity. Second, small numbers of bacteria are most likely reaching the hemolymph in each wave, as inferred from the small numbers of *Sm* CFUs scored in the hemolymph after oral infections (Fig. 14a), which might not trigger a measurable PO activity. This is in contrast to bacterial injections where the time of hemolymph infection and the dose of introduced bacteria can be optimized to trigger a measurable PO response [386, 515]. In *Drosophila*, melanization was shown to be essential for immune defense against septic infections with a small dose of *S. aureus* [355]. However, the fact that silencing TEP1, a key upstream regulator of the mosquito melanization response [300, 311, 386] did not alter mosquito susceptibility to *Sm* oral infection, and that PPO6 and several CLIPs were downregulated after feeding on *Sm*, suggest that melanization may not play an essential role in this route of infection.

GO enrichment analysis identified a total of 10 CLIP genes (CLIPC4, CLIPB4, CLIPB1, CLIPC9, CLIPB13, CLIPA8, CLIPA4, CLIPA6, CLIPA1 and CLIPA7) enriched in the midgut. The fact that all these genes were downregulated suggests that *Sm* oral infection may suppress the melanization response regulated by several of these CLIPs. Among the enriched downregulated CLIPs, CLIPB4 and CLIPC9, both catalytic clips, are involved in the melanization of *P. berghei* ookinetes in refractory mosquito

backgrounds [383, 516], while CLIPA8 and CLIPA7 are non-catalytic CLIPs that act as positive and negative regulators of *Plasmodium* melanization, respectively [383]. The melanization response to fungal infections also requires CLIPA8 [311], while both CLIPA8 and CLIPC9 play an essential role in the melanization response to bacterial infections [384, 386, 516]. CLIPA1, CLIPA4, CLIPA6 and CLIPB1 do not seem to be involved in *Plasmodium* melanization [383], whereas the roles of CLIPC4 and CLIPB13 in the melanization response remains to be elucidated. Of note, the differential regulation of CLIPs in the midgut is most likely attributed to hemocytes attached to the midgut surface and not to the midgut epithelium *per se*, as insect CLIPs are mainly expressed in hemocytes and fat body cells [350, 498]. Indeed, several of the over-represented CLIPs in our study including CLIPA7, CLIPA8, CLIPB1, CLIPB4, CLIPB13, CLIPC4, and CLIPC9, were among the genes identified in transcriptomic studies of mosquito hemocytes [104, 517]. Also, PPO6 which is hemocyte-specific [517, 518], was among the DETs identified in mosquito midguts in response to *Sm* oral infections (See appendix, Table 1), further indicating that some of the immunity genes identified in the midgut transcriptome are attributed to midgut-attached hemocytes rather than to the midgut epithelium. Indeed, there is evidence that contact between midgut epithelial cells and the gut microbiota which occurs during *Plasmodium* midgut invasion, initiates systemic immune priming by triggering hemocyte differentiation and their attraction to the midgut surface where they present anti-microbial activities including complement activation [461-463]. Subsequent studies employing confocal microscopy and immunostaining of candidate CLIPs will be required to determine whether these are expressed by gut epithelial cells to prime the humoral immune response in the hemolymph or by hemocytes attached to it as a result of immune

priming by invading *Serratia* cells.

### E. Neither the Imd pathway nor phagocytosis seems to be a determinant of mosquito susceptibility to oral *Sm* infections

*Rel2* silencing did not affect mosquito survival to oral *Sm* infections, suggesting that the Rel2/Imd pathway may be either non-essential for defense against *Sm* invasion of the hemolymph following an oral infection or that the pathway is not activated through this route. In *Drosophila*, *Sm* was sensitive to the local Imd response in the gut but failed to activate the systemic response in the fat body after crossing the gut epithelium into the hemolymph [440]. Currently, it is not possible to accurately score the activation of the mosquito Rel2 pathway due to the absence of a specific gene-expression signature associated with this pathway. However, in our RNA-seq analysis, several immunity genes that are known to be at least partially regulated by Rel2, such as TEP1, APL1, several CLIPs and FBNs among others [68, 184], were not upregulated neither in the midgut nor in the abdomens after oral *Sm* infection, suggesting that the Imd pathway may not be activated through this route. Phagocytosis is an essential determinant of *Drosophila* susceptibility to *Sm* oral infection [440]. However, this does not seem to be the case in *A. gambiae*, since the treatment of mosquitoes with cytochalasin D did not alter their susceptibility to oral *Sm* infection. This result may suggest either the existence of functional redundancy among different branches of the immune response in this route of infection or that phagocytosis may not be essential when small numbers of bacteria are present in the hemolymph. Indeed, a recent study in *Drosophila* revealed that hemocyte-deficient flies did not succumb to a low dose *S. aureus* infection but a high dose did compromise their survival [355].

## F. Abdomen and midgut transcriptional responses after *Sm* oral and septic infections

### *1. Transcriptional responses following Sm oral infection*

Our RNA-seq analysis identified a limited number of DETs in the midguts after *Sm* oral infection, specifically at the late 24 h time point. This agrees with a previous microarray-based study that compared the transcriptional responses in the guts of antibiotic treated-mosquitoes at 3 days after *Sm* oral infection with those of antibiotic treated uninfected mosquitoes [441]. Another microarray-based study in *A. gambiae* that compared the gut transcriptomes of antibiotic treated and untreated mosquitoes also identified a limited set of differentially expressed genes [127]. On the other hand, oral infections in *Drosophila* trigger dramatic changes in the gut transcriptome [519, 520]. This discrepancy can be explained by the different nature of the food source of both species; while *Drosophila* feeds mainly on fermented and rotten fruits rich in yeast and bacteria [521, 522], *A. gambiae* mosquitoes feed mainly on human blood which is sterile. It should also be noted that in oral infections the number of DETs increased in the abdomens with time, whereas, the inverse was observed in midguts. This DETs pattern in abdomens may be due to the continuous crossing of *Sm* from the gut into the hemolymph triggering physiological responses in the fat body and/or hemocytes attached to it, or due to signaling between the gut epithelium on one hand and the fat body and hemocytes on the other. Inter-organ communication has been mainly studied in *Drosophila* whereby pathogen-infected intestinal cells signal to hemocytes, which in turn regulate intestinal regeneration [464, 465]. There is also evidence for signaling between the gut and fat body in *Drosophila* to regulate energy homeostasis [523, 524].

Our RNA-seq analysis also revealed that the response to *Sm* oral infections is more pronounced in the midgut, and it becomes even more pronounced when the analysis is restricted to transcripts that uniquely respond to feeding on *Sm*. Most of these transcripts are associated with biological processes related to protein translation, cell cycle and DNA repair, which may not be surprising since *Sm* infection of the *Drosophila* gut was shown to trigger significant damage to the gut epithelium that alters cell morphology and physiology [525]. Epithelial damage of the gut and enhancement of gut physiological responses associated with stress, cell renewal and proliferation have been also observed in *Drosophila* intestinal infections with *Erwinia corotovora* [519].

### *2. The wounding process triggers complex physiological responses in insects*

Only 4 genes showed an expression pattern unique to *Sm* injection suggesting that most of the transcriptional response is triggered by the wounding process *per se*. This was not surprising, since a previous study by Dimopoulos *et al.* showed that the predominant transcriptional responses triggered by septic and sterile injury in the refractory L3-5 mosquito strain were shared, suggesting that most of these responses are attributed to injury and/or wound healing [125]; injury-specific transcriptional responses were dominated by functional groups pertaining to carbohydrate metabolism, whereas septic infection was dominated by immunity genes. Interestingly, a separate study showed that wounding of *A. gambiae* mosquitoes by the injection of water or dsRNA triggers the killing of *P. falciparum* parasites in a TEP1-dependent manner [526]. To better understand the relationship between wounding and immune defense to *Plasmodium*, the authors performed a genome wide analysis of the transcriptional response to wounding in adult *A. gambiae* mosquitoes and identified 53 genes with

statistically significant regulation that were enriched mainly in genes involved in proteolysis-related processes including several CLIP genes. Wounding also triggered the expression of several immunity genes with known anti-*Plasmodium* roles such as TEP1, LRIM1, APL1C and FBN9 among others [527]. The fact that wounding triggers the expression of several mosquito immunity genes explains most likely why no immunity genes showed an expression pattern unique to *Sm*_inj in our study. The complex physiological responses triggered by wounding in other insects [reviewed in [528]] lend further support to our conclusion. In *Drosophila* for instance, where the wound healing process is best characterized, cellular responses mediated by hemocytes, epithelial cells and fat body cells act in concert with humoral factors including, hemolectin and fondue to seal the wound, clear tissues debris and initiate soft clot formation that becomes eventually melanized by the action of crystal cell-derived phenoloxidase [529-537]. Interestingly, fat body cells were also shown to secrete antimicrobials peptides locally to protect from wound infection [530].

### *3. Crosstalk between immunity and metabolism in vertebrates and invertebrates*

Our transcriptomic analysis revealed that metabolic genes are the most represented functional class of all DETs in abdomens and midguts from all treatments. Knowing that metabolism is at the core of all biological processes, this result comes as no surprise. There is currently ample evidence in mammals, specifically from studies in mice, that cellular metabolism shapes the activation and differentiation of myeloid and lymphoid immune cells during infection [538, 539]. This relation has been particularly addressed in macrophages, whereby proinflammatory macrophages of the M1 type exhibit a metabolic shift to aerobic glycolysis associated with the production of nitric

oxide, reactive oxygen species and prostaglandins; whereas, M2 macrophages exhibit a shift towards oxidative phosphorylation and increased dependency on a complete Krebs cycle fueled by glucose, fatty acids and glutamine [reviewed in [540]]. Our knowledge of immunometabolism in mosquitoes is fragmented, with emerging evidence pointing toward a cross-talk between metabolic genes or metabolic signaling pathways and immune defense processes. In *A. stephensi*, *P. falciparum* infection was shown to induce the expression of insulin-like peptides that favor parasite development by suppressing NF-κB signaling pathway in the midgut and by triggering metabolic shifts in this tissue independent of NF-κB [541]. Human insulin ingested by mosquitoes during blood feeding was also shown to enhance *Plasmodium* development by inhibiting NF-κB-dependent immune responses [542]. This reciprocal effect between immunity and insulin signaling have been also reported in *Drosophila* [543]. Another example of cross-talk between immunity and metabolism in mosquitoes is the finding that Lipophorin, a multifunctional carrier involved in lipid transport and metabolism, and its receptor are upregulated in *Ae. aegypti* mosquitoes following infection with Gram-positive bacteria and fungi in a Toll/Rel1 dependent manner [544]. In *A. gambiae*, *Apolipophorin-II/I* was shown to control *TEP1* expression during systemic infections with *E. coli* and *B. bassiana* [302]. Metabolic decisions may also influence the outcome of mosquito infection with microbes independent of immunity, and this has been mainly studied in the context of *Plasmodium* infections. For instance, the susceptibility of refractory and susceptible strains of *A. gambiae* to infection with *P. berghei* was shown to be influenced by broad metabolic differences between these strains, whereby the refractory strain exhibits rapid utilization of lipids, impaired mitochondrial respiration, and increased glycolytic activity leading to higher ROS

production that is toxic to malaria parasites [545]. In a more recent study, Lampe *et al.* elegantly showed that the timely expression of the blood-meal inducible miR-276 finely regulates the rate of amino acid catabolism, terminating the investment in reproductive processes and providing excess resources for the sporogonic development of *P. falciparum* [546]. In a similar context, it would be interesting to determine whether metabolic shifts induced by blood feeding would influence mosquito resistance to bacterial and fungal infections and through what mechanisms. It was interesting to note that genes involved in oxidative phosphorylation (OXPHOS) were over-represented in our KEGG pathway enrichment analysis, and all were downregulated in the midgut after feeding on *Sm*. This metabolic shift away from OXPHOS (a catabolic process) may reflect increased dependency on anabolic processes such as aerobic glycolysis that would be required to promote midgut tissue repair in response to the damage triggered by *Sm* intestinal infection [440]. Tissue repair processes are known to be anabolic in nature and contribute to host tolerance to infection [539, 547]. Our transcriptomic analysis also revealed that the abdomen transcriptome was substantially larger in *Sm* oral infections relative to injections, at all three time points (compare Figs. 8a and 8c), despite the fact that injections resulted eventually in higher loads of *Sm* in the hemolymph. While these results may reflect different adaptive strategies of the host in response to different routes of infection with the same microbe, they possibly pinpoint also to a potential key role of the midgut epithelium in priming immune and non-immune physiological responses in the fat body and hemocytes that should act in concert to control hemolymph infections.

## G. Perspectives

In conclusion, we provide evidence using gene silencing and transcriptomic analysis that the dynamics of immune defense to bacterial hemolymph infections through the midgut are different from those of hemolymph infections established by septic injections. The key difference between both routes is that the first involves the gut as a natural route towards establishing systemic infection while the second utilizes the more artificial or ″naturally less common″ wounding process to do so. Indeed, ingested pathogens need to avoid evacuation, resist oxidative stress, and/or breach the epithelial gut barrier to establish infection in their hosts [445, 548-550]. On the other hand, these layers of immunity are bypassed in systemic infections [551] leading to virulence at much lower doses [552] and triggering melanization responses that are not observed in oral infections [553]. Indeed, one should keep in mind that oral infections may induce a systemic response [549, 552, 554], although not necessarily always [440]. Being at the front line of microbial defense, it is not surprising that the midgut epithelium, in addition to its classical evolutionary conserved role in local immune defense through its physical impermeability and chemical defenses, also plays an important role in priming physiological responses in distant organs that provide the host with better resistance and tolerance in case the microbe succeeds in crossing this barrier to establish a systemic infection. The nature of these protective physiological responses and how they are primed by the midgut epithelium remain largely unknown.

# CHAPTER V

# MATERIALS AND METHODS

## A. *Anopheles gambiae* rearing

All experiments were performed with adult female *A. gambiae* G3 strain mosquitoes. Mosquitoes were maintained at 27 (±1) °C and 75 (±5) % humidity with 12-hour day-night cycle. Larvae were reared in 752 $cm^2$ plastic pans at a density of approximately 150 larvae per pan and given tropical fish food. Freshly emerged adult mosquitoes were collected from larval pans using a vacuum collector, maintained on 10% sucrose and given Bagg Albino (BALB/c) mice blood (mice were anesthetized with ketamine) for egg laying.

## B. Double-stranded RNA synthesis and gene silencing by RNA interference

For dsRNA production, gene specific DNA amplicons flanked by T7 promoter sequences were produced using T7- labelled primers (listed in Table 3) by PCR amplification of cDNA extracted from mosquitoes previously infected with *E. coli* or *S. aureus* or otherwise from plasmids containing full length cDNA sequence according to the following protocol: 95°C for 3min; 95°C for 40s; 60°C for 1min; 72°C for 1min; cycles 30-35 times; 72°C for 10min; 4°C forever. Illustra GFX PCR DNA Gel Band Purification Kit (GE Healthcare) was used to purify the PCR amplicons, according to the manufacturer's instructions.

dsRNA synthesis was performed using the T7 RiboMax Express Large Scale RNA production system (Promega) according to the manufacturer's instructions, and dsRNAs were purified as previously described [278]. Briefly, transcription reaction was allowed

to run overnight 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 3.5-4 μg/μl. In vivo gene silencing was performed as previously described [278]. Briefly, mosquitoes were microinjected with 69nl of a 4 μg/μl solution of gene-specific dsRNA, and allowed to recover for 3-4 days before proceeding with Sm infections. The efficiency of gene silencing by RNAi for TEP1 and CTL4 was quantified by Western blot in hemolymph extracts of naive mosquitoes at 3 days after dsRNA injection, as previously described [155], using the following dilution of primary antibodies: rabbit αTEP1 (1:1000) and rabbit αCTL4 (1:1000). Rabbit αSRPN3 (1:1000) was used to control for loading. The silencing efficiency of *Rel1* and *Rel2* was determined by qRT-PCR in naïve mosquitoes at 3 days after dsRNA injection.

### C. RNA Extraction and Real-Time PCR

Around 20 mosquitoes per genotype were stored in 300μl TRIzol Reagent (Invitrogen). Nucleic acids were extracted with chloroform (1:5 chloroform to TRIzol ratio) and contaminant genomic DNA was removed by 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. First strand cDNA was synthesized by reverse transcription using the iScript$^{TM}$ cDNA Synthesis kit (BioRad) according to the manufacturer's protocol. In brief, the equivalent of 1 μg of RNA was mixed with 4 μl 5x iScript reaction mix and 1 μl iScript reverse transcriptase and nuclease-free water was added to a total volume of 20 μl. The reverse transcription reaction mix was incubated for 5min at 25°C followed by 30 min at 42°C and a final incubation for 5min at 85°C, and the produced cDNA templates were used in real-time

PCR. In brief, cDNA samples were diluted 5 or 10x, and in a 96-well microtiter plate 5µl of the diluted cDNA was mixed with 12.5µl SYBR Green JumpStart $^{TM}$ Taq ReadyMix $^{TM}$, 2.5 µl nuclease-free-water, 2.5 µl forward primer and 2.5 µl reverse primer (previously standardized by qRT-PCR). The plate was properly sealed, centrifuged at low speed for 1 min at 4°C, and placed in a CFx96 Systems light cycler machine (95$^{O}$C for 3mins ; 95$^{O}$C for 10s ; cycles 40 times ; 60$^{O}$C for 30s). Relative gene expression was normalized relative to the mosquito gene encoding the ribosomal protein S7 and calculated using the comparative CT method after checking for the efficiency of target amplification. The primers used in qRT-PCR are listed in Table 3.

**D. Mosquito infections with *Serratia marcescens*, and survival assays**

Mosquito oral infections with *Sm* were performed by allowing mosquitoes to feed continuously on a sugar solution containing *Sm* that was prepared as follows. DsRed-expressing, gentamycin-resistant *Sm* strain DB11 [440] cultured exponentially at 37 °C was washed with PBS then diluted in a sterile 3% sucrose solution to a final $OD_{600}$=1. Mosquitoes that fed on *Sm* containing sugar pads were sorted out at 24 h after feeding on *Sm* with the help of a food colorant added to the sugar solution, and used in subsequent experiments. Mosquitoes were maintained on *Sm* containing sugar pads for the duration of the experiment. To determine whether our mosquito colony naturally contains *Sm*, DNA was extracted from a pool of 10 midguts dissected from either sugar fed adult female mosquitoes or mosquitoes that have been feeding on *Sm* DB11 strain for 24 hours, using the DNeasy Blood and Tissue Kit (Qiagen). A 100 ng of extracted DNA per sample was used to amplify a 175 bp amplicon of the LuxS gene involved in quorum sensing using *Sm* LuxS-specific primers, For, 5'-TGCCTGGAAAGCGGCGATGG-3' and Rev, 5'-CGCCAGCTCGTCGTTGTGGT-3'

[555], according to the following program (45 sec at 95°C; 60 sec at 66°C; 60 sec at 72°C) for 33 cycles. As internal control, we PCR amplified a 298 bp amplicon of the gene encoding *A. gambiae* ribosomal protein S7 (*Ag*_S7) using primers, For, 5'-AGAACCAGCAGACCACCATC-3' and Rev, 5'-GCTGCAAACTTCGGCTATTC-3', according to the following program (45 sec at 95°C; 60 sec at 60°C; 60 sec at 72°C) for 33 cycles. Amplicons were separated on a 1.2 % agarose gel, stained with Ethidium bromide and analyzed on ChemiDoc MP (BioRad).

Septic infections with *Sm* were performed by the intrathoracic microinjection of dsRNA-treated mosquitoes with a suspension of DsRed-expressing gentamycin-resistant *Sm* strain DB11 in PBS ($OD_{600}$=0.0005). Mosquitoes treated with dsRNA specific to the β-galactosidase gene (ds*LacZ*) served as control. Mosquito survival was scored over a period of 8-10 days after *Sm* septic or oral infections. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test. Experiments were repeated at least 3 times using different mosquito and bacterial batches. At least 50 mosquitoes were utilized per sample per experiment.

**E. Scoring *Sm* colony forming units (CFUs) in infected mosquitoes**

To determine *Sm* CFUs in whole mosquitoes following septic injections, batches of 8 mosquitoes each per genotype were grinded using a micropestle in 400µl Luria Bertani (LB) Broth at 24 h after *Sm* injections. The homogenate was serially diluted in LB medium. After overnight culturing at 37 °C on LB agar supplemented with Gentamycin, CFUs were scored under a fluorescence stereomicroscope.

To determine *Sm* CFUs in the hemolymph, hemolymph was collected 72 h post oral infections with *Sm* by perfusion as follows. Briefly, mosquitoes were perfused with 5 µl

of PBS injected into the thorax using a Nanoject II (Drumond Scientific) nanoinjector and perfused hemolymph was collected from a small incision made in the 3rd abdominal segment. Hemolymph was collected in sterile ice-cold PBS from batches of 4 or 5 mosquitoes each, serially diluted, then plated on LB agar with the appropriate antibiotic. CFUs were scored after culturing overnight at 37 °C on LB agar supplemented with Gentamycin. Statistical significance was calculated using the Mann-Whitney test in GraphPad Prism software (version 6.0). Medians were considered significantly different if $P<0.05$.

For the bacterial fitness experiment, ds*CTL4* and ds*LacZ* (control) mosquitoes were injected with *Sm* prepared from a fresh bacterial culture ($OD_{600}$ = 0.0005) or *Sm* collected by hemolymph perfusion from wildtype mosquitoes that have been feeding on *Sm* for 24 hrs. Mosquitoes injected with hemolymph perfusate received 4, 207, and 331 *Sm* CFUs in the three independent biological experiments performed. *Sm* proliferation in injected mosquitoes was scored by homogenizing batches of 8 whole mosquitoes each in LB medium at 24 h after *Sm* injection. The homogenate was serially diluted in LB medium. CFUs were scored under a fluorescence stereomicroscope after culturing overnight at 37 °C on LB agar supplemented with Gentamycin.

**F. Mosquito treatment with Cytochalasin D**

Cytochalasin D was dissolved in DMSO to make a 1 mg/ml stock solution from which a 62.5 μg/ml (120 μM) working solution in PBS was prepared. Each mosquito was injected with 69 nl of the working solution. Control groups included mosquitoes injected with PBS only and those injected with 6.25 % DMSO in PBS. Mosquitoes injected with PBS, DMSO, or Cytochalasin D were allowed to recover for 6 h before feeding on *Sm* ($OD_{600}$=1), and for 24 h before injection with *Sm* ($OD_{600}$=0.0005).

## G. RNA extraction, library preparation and sequencing

RNA was extracted from midguts and abdomens dissected from untreated control female mosquitoes (fed on 3% sugar solution), and from female mosquitoes treated by oral *Sm* feeding, *Sm* injection and sterile PBS injection using a hybrid modified Trizol/RNeasy protocol (Qiagen). Untreated mosquitoes served as control for mosquitoes fed on *Sm*, and PBS-injected mosquitoes as control for *Sm*-injected mosquitoes. By including these respective controls we would be assessing transcriptional responses that are *Sm* specific in each route, which allows us to focus on genes regulated by *Sm* itself and not secondary to the infection procedure. The abdomen specimen refers to the whole abdomen excluding the gut in addition to the malpighian tubules and ovaries which were pulled out with the gut during dissection. Among treated mosquitoes, RNA was extracted from the indicated tissues at 6 h, 12 h and 24 h post treatment. RNA quantification was performed using the Qubit RNA HS Assay and quality check procedures via AATI Fragment Analyzer. QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen) was used for construction of 3' end RNA-seq libraries. Libraries were checked with Qubit DNA Assay kit and AATI Fragment Analyzer again before pooling and sequencing. Illumina NextSeq 500 platform with standard protocol for 75 bp single-end read sequencing was utilized to sequence libraries at the Cornell Life Sciences Sequencing core facility. Three to six million reads were obtained per sample, which is equivalent approximately to a 20x or more coverage of the transcriptome. Quality control of raw reads was performed with FastQC, followed by trimming of the reads by BBMap (https://sourceforge.net/projects/bbmap/), and then mapping to the *A. gambiae* transcriptome (AgamP4.12) using Salmon [556].

## H. Differential gene expression and gene ontology

Differential expression was analyzed on the transcript level using Bioconductor package DESeq2 [557]. A model with two categorical variables was fitted, one variable for the replicate and a second variable that contained a separate level for each of the 18 combinations of tissue (abdomen or midgut), time (6 h, 12 h, and 24 h), and treatment (*Sm* oral infection, *Sm* injection, and PBS injection), plus a level for the untreated control (only sugar fed) at time zero. Differential expression was analyzed by fitting a generalized linear model and testing for a significant difference in coefficients for treatment and control. This analysis was performed within each combination of time and tissue by comparing read counts between *Sm* oral infection and the untreated control, between PBS injection and the untreated control, and between *Sm* injection and PBS injection of the same time point. The transcript-specific P-values for differential expression were adjusted for a false discovery rate, and only transcripts with a false discovery rate below 0.05 were labeled as differentially expressed. Genes with at least one differentially expressed transcript were labelled differentially expressed. To identify transcripts whose expression changes significantly and uniquely in response to oral feeding of *Sm* relative to untreated control, and those whose expression changes significantly and uniquely in response to *Sm* injection relative to PBS injection, we took all transcripts that are differentially expressed in each treatment of interest (P-value adjusted for false discovery rate $< 0.05$) and removed all transcripts that showed differential expression in the same direction in any of the other comparisons, either according to false-discovery rate adjusted P-value (P-value $< 0.05$) or according to fold change (fold change $> 1.5$). The fold change criterion was included to be confident that the remaining transcripts are actually treatment-specific. Enrichment of differentially

expressed genes was tested for each of treatment-control comparison according to four classifications, namely gene ontology terms from the molecular function and biological process ontology, KEGG pathways, and gene families. The enrichment tests used Wallenius non-central hypergeometric distribution to account for transcript-length dependent bias for detecting differential expression as implemented in the R package *goseq* [558]. The false discovery rate was calculated by selecting all groups from all four classifications that contain more than one significantly differentially expressed gene in any of the comparisons of differential expression, and applying the Benjamini-Hochberg correction [559] to the enrichment P-values of all these groups. Only terms with a false discovery rate below 0.05 were reported.

*Table 3. Primers used for dsRNA production and qRT-PCR*

| Gene | Primers used for dsRNA synthesis (T7 promoter sequence underlined) | References |
|---|---|---|
| *LacZ* | For: 5'-<u>TAATACGACTCACTATAGGG</u>AGAATCCGACGGGTTGTTACT-3'<br>Rev: 5'-<u>TAATACGACTCACTATAGGG</u>CACCACGCTCATCGATAATTT-3' | [560] |
| *TEP1* (AGAP010815) | For: 5'-<u>TAATACGACTCACTATAGGG</u>TTTGTGGGCCTTAAAGCGCTG-3'<br>Rev: 5'-<u>TAATACGACTCACTATAGGG</u>ACCACGTAACCGCTCGGTAAG-3' | [295] |
| *CTL4* (AGAP005335) | For: 5'-<u>TAATACGACTCACTATAGGG</u>GTTAGCAGCATTGGGATTACCCT-3'<br>Rev: 5'-<u>TAATACGACTCACTATAGGG</u>GAAGTCGCAACCCAGCTCATTGT-3' | [386] |
| *Rel1* (AGAP009515) | For: 5'-<u>TAATACGACTCACTATAG</u>ATCAACAGCACGACGATGAG-3'<br>Rev: 5'-<u>TAATACGACTCACTATAG</u>TCGAAAAAGCGCACCTTAAT-3′ | [230] |
| **Gene** | **Primers used for qRT-PCR** | |
| *Rel1* | For: 5'- CCAACCTCGATCCGGTGTTCA-3'<br>Rev: 5'-TAGGTCGGTCGTGGAAAGTGA-3' | |

| | | |
|---|---|---|
| *Rel2* | For: 5'-GCCATTCCGGAAGGTCAAGA-3'<br>Rev: 5'-AATGTCCGGATGATGGGCTGA-3' | |
| *S7* (AGAP010592) | For: 5'-GTGCGCGAGTTGGAGAAGA<br>Rev: 5'-ATCGGTTTGGGCAGAATGC | [276] |

# BIBLIOGRAPHY


1. Nerlich, A.G., et al., *Plasmodium falciparum in ancient Egypt.* Emerg Infect Dis, 2008. **14**(8): p. 1317-9.
2. Cui, L., et al., *Antimalarial Drug Resistance: Literature Review and Activities and Findings of the ICEMR Network.* Am J Trop Med Hyg, 2015. **93**(3 Suppl): p. 57-68.
3. Liu, N., *Insecticide resistance in mosquitoes: impact, mechanisms, and research directions.* Annu Rev Entomol, 2015. **60**: p. 537-59.
4. Hay, S.I., et al., *Developing global maps of the dominant anopheles vectors of human malaria.* PLoS Med, 2010. **7**(2): p. e1000209.
5. Antinori, S., et al., *Biology of human malaria plasmodia including Plasmodium knowlesi.* Mediterr J Hematol Infect Dis, 2012. **4**(1): p. e2012013.
6. Caldelari, R., et al., *Transcriptome analysis of Plasmodium berghei during exo-erythrocytic development.* Malar J, 2019. **18**(1): p. 330.
7. Guleria, V. and V. Jaiswal, *Comparative transcriptome analysis of different stages of Plasmodium falciparum to explore vaccine and drug candidates.* Genomics, 2020. **112**(1): p. 796-804.
8. Howick, V.M., et al., *The Malaria Cell Atlas: Single parasite transcriptomes across the complete Plasmodium life cycle.* Science, 2019. **365**(6455).
9. Billker, O., et al., *Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito.* Nature, 1998. **392**(6673): p. 289-92.
10. Billker, O., et al., *The roles of temperature, pH and mosquito factors as triggers of male and female gametogenesis of Plasmodium berghei in vitro.* Parasitology, 1997. **115 ( Pt 1)**: p. 1-7.

11. Lensen, A.H., et al., *Leukocytes in a Plasmodium falciparum-infected blood meal reduce transmission of malaria to Anopheles mosquitoes.* Infect Immun, 1997. **65**(9): p. 3834-7.

12. Margos, G., et al., *Interaction between host complement and mosquito-midgut-stage Plasmodium berghei.* Infect Immun, 2001. **69**(8): p. 5064-71.

13. Simon, N., et al., *Malaria parasites co-opt human factor H to prevent complement-mediated lysis in the mosquito midgut.* Cell Host Microbe, 2013. **13**(1): p. 29-41.

14. Ramiro, R.S., et al., *Sex and death: the effects of innate immune factors on the sexual reproduction of malaria parasites.* PLoS Pathog, 2011. **7**(3): p. e1001309.

15. Grotendorst, C.A., et al., *Complement effects on the infectivity of Plasmodium gallinaceum to Aedes aegypti mosquitoes. I. Resistance of zygotes to the alternative pathway of complement.* J Immunol, 1986. **136**(11): p. 4270-4.

16. Tsuboi, T., et al., *Murine complement reduces infectivity of Plasmodium yoelii to mosquitoes.* Infect Immun, 1995. **63**(9): p. 3702-4.

17. Smith, R.C., J. Vega-Rodriguez, and M. Jacobs-Lorena, *The Plasmodium bottleneck: malaria parasite losses in the mosquito vector.* Mem Inst Oswaldo Cruz, 2014. **109**(5): p. 644-61.

18. Wang, S. and M. Jacobs-Lorena, *Genetic approaches to interfere with malaria transmission by vector mosquitoes.* Trends Biotechnol, 2013. **31**(3): p. 185-93.

19. Tizifa, T.A., et al., *Prevention Efforts for Malaria.* Curr Trop Med Rep, 2018. **5**(1): p. 41-50.

20. Lim, S.S., et al., *Net benefits: a multicountry analysis of observational data examining associations between insecticide-treated mosquito nets and health outcomes.* PLoS Med, 2011. **8**(9): p. e1001091.

21. Atieli, H.E., et al., *Insecticide-treated net (ITN) ownership, usage, and malaria transmission in the highlands of western Kenya.* Parasit Vectors, 2011. **4**: p. 113.

22. N'Guessan, R., et al., *Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin.* Emerg Infect Dis, 2007. **13**(2): p. 199-206.

23. Strode, C., et al., *The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African anopheline mosquitoes: systematic review and meta-analysis.* PLoS Med, 2014. **11**(3): p. e1001619.

24. Pluess, B., et al., *Indoor residual spraying for preventing malaria.* Cochrane Database Syst Rev, 2010(4): p. CD006657.

25. Okullo, A.E., et al., *Malaria incidence among children less than 5 years during and after cessation of indoor residual spraying in Northern Uganda.* Malar J, 2017. **16**(1): p. 319.

26. Raouf, S., et al., *Resurgence of Malaria Following Discontinuation of Indoor Residual Spraying of Insecticide in an Area of Uganda With Previously High-Transmission Intensity.* Clin Infect Dis, 2017. **65**(3): p. 453-460.

27. WHO, *Global Technical Strategy for Malaria 2016–2030*. 2015: Geneva.

28. West, P.A., et al., *Indoor residual spraying in combination with insecticide-treated nets compared to insecticide-treated nets alone for protection against malaria: a cluster randomised trial in Tanzania.* PLoS Med, 2014. **11**(4): p. e1001630.

29. Stone, C.M., S.W. Lindsay, and N. Chitnis, *How effective is integrated vector management against malaria and lymphatic filariasis where the diseases are transmitted by the same vector?* PLoS Negl Trop Dis, 2014. **8**(12): p. e3393.

30. Thome, R.C., H.M. Yang, and L. Esteva, *Optimal control of Aedes aegypti mosquitoes by the sterile insect technique and insecticide.* Math Biosci, 2010. **223**(1): p. 12-23.

31. Benedict, M.Q. and A.S. Robinson, *The first releases of transgenic mosquitoes: an argument for the sterile insect technique.* Trends Parasitol, 2003. **19**(8): p. 349-55.

32. Klassen, W., *Introduction: development of the sterile insect technique for African malaria vectors.* Malar J, 2009. **8 Suppl 2**: p. I1.

33. *Sterile insect technique: principles and practice in area-wide integrated pest management.* Choice: Current Reviews for Academic Libraries, 2006. **43**(9): p. 1851-1852.

34. Nolan, T., et al., *Developing transgenic Anopheles mosquitoes for the sterile insect technique.* Genetica, 2011. **139**(1): p. 33-9.

35. Derek W. Dunn, P.A.F., *The Sterile Insect Technique-an introduction*, in *Entomologia Experimentalis et Applicata*. 2017. p. 151-154.

36. Atzeni, M.G., et al., *Comparison of the predicted impact of a screwworm fly outbreak in Australia using a growth index model and a life-cycle model.* Med Vet Entomol, 1994. **8**(3): p. 281-91.

37. Knipling, E.F., et al., *Genetic control of insects of public health importance.* Bull World Health Organ, 1968. **38**(3): p. 421-38.

38. Bouyer, J. and T. Lefrancois, *Boosting the sterile insect technique to control mosquitoes.* Trends Parasitol, 2014. **30**(6): p. 271-3.

39. Juan-Blasco, M., et al., *Estimating SIT-driven population reduction in the Mediterranean fruit fly, Ceratitis capitata, from sterile mating.* Bull Entomol Res, 2014. **104**(2): p. 233-42.

40. Abd-Alla, A.M., et al., *Improving Sterile Insect Technique (SIT) for tsetse flies through research on their symbionts and pathogens.* J Invertebr Pathol, 2013. **112 Suppl**: p. S2-10.

41. Vreysen, M.J., et al., *Glossina austeni (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique.* J Econ Entomol, 2000. **93**(1): p. 123-35.

42. Gilles, J.R., et al., *Towards mosquito sterile insect technique programmes: exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes.* Acta Trop, 2014. **132 Suppl**: p. S178-87.

43. Dame, D.A., et al., *Historical applications of induced sterilisation in field populations of mosquitoes.* Malar J, 2009. **8 Suppl 2**: p. S2.

44. Helinski, M.E., A.G. Parker, and B.G. Knols, *Radiation biology of mosquitoes.* Malar J, 2009. **8 Suppl 2**: p. S6.

45. Lees, R.S., et al., *Back to the future: the sterile insect technique against mosquito disease vectors.* Curr Opin Insect Sci, 2015. **10**: p. 156-162.

46. LePage, D.P., et al., *Prophage WO genes recapitulate and enhance Wolbachia-induced cytoplasmic incompatibility.* Nature, 2017. **543**(7644): p. 243-247.

47. Yen, J.H. and A.R. Barr, *New hypothesis of the cause of cytoplasmic incompatibility in Culex pipiens L.* Nature, 1971. **232**(5313): p. 657-8.

48. Hughes, G.L., et al., *Native microbiome impedes vertical transmission of Wolbachia in Anopheles mosquitoes.* Proc Natl Acad Sci U S A, 2014. **111**(34): p. 12498-503.

49. Jeffries, C.L., et al., *Novel Wolbachia strains in Anopheles malaria vectors from Sub-Saharan Africa.* Wellcome Open Res, 2018. **3**: p. 113.

50. Rossi, P., et al., *Mutual exclusion of Asaia and Wolbachia in the reproductive organs of mosquito vectors.* Parasit Vectors, 2015. **8**: p. 278.

51. Bian, G., et al., *Wolbachia invades Anopheles stephensi populations and induces refractoriness to Plasmodium infection.* Science, 2013. **340**(6133): p. 748-51.

52. Boissiere, A., et al., *Midgut microbiota of the malaria mosquito vector Anopheles gambiae and interactions with Plasmodium falciparum infection.* PLoS Pathog, 2012. **8**(5): p. e1002742.

53. Hurwitz, I., et al., *Paratransgenic control of vector borne diseases.* Int J Biol Sci, 2011. **7**(9): p. 1334-44.

54. Drexler, A.L., Y. Vodovotz, and S. Luckhart, *Plasmodium development in the mosquito: biology bottlenecks and opportunities for mathematical modeling.* Trends Parasitol, 2008. **24**(8): p. 333-6.

55. Whitten, M.M., S.H. Shiao, and E.A. Levashina, *Mosquito midguts and malaria: cell biology, compartmentalization and immunology.* Parasite Immunol, 2006. **28**(4): p. 121-30.

56. SibaoWang, M.-L., *Paratransgenesis Applications: Fighting Malaria With Engineered Mosquito Symbiotic Bacteria*, in *Arthropod Vector: Controller of Disease Transmission*. 2017. p. 219-234.

57. Riehle, M.A., et al., *Using bacteria to express and display anti-Plasmodium molecules in the mosquito midgut.* Int J Parasitol, 2007. **37**(6): p. 595-603.

58. Wang, S., et al., *Fighting malaria with engineered symbiotic bacteria from vector mosquitoes.* Proc Natl Acad Sci U S A, 2012. **109**(31): p. 12734-9.

59. Wang, S., et al., *Driving mosquito refractoriness to Plasmodium falciparum with engineered symbiotic bacteria.* Science, 2017. **357**(6358): p. 1399-1402.

60. Shane, J.L., et al., *Blood meal-induced inhibition of vector-borne disease by transgenic microbiota.* Nat Commun, 2018. **9**(1): p. 4127.

61. Wilke, A.B. and M.T. Marrelli, *Paratransgenesis: a promising new strategy for mosquito vector control.* Parasit Vectors, 2015. **8**: p. 342.

62. Galizi, R., et al., *A synthetic sex ratio distortion system for the control of the human malaria mosquito.* Nat Commun, 2014. **5**: p. 3977.

63. Hammond, A., et al., *A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae.* Nat Biotechnol, 2016. **34**(1): p. 78-83.

64. Ghosh, A.K., P.E. Ribolla, and M. Jacobs-Lorena, *Targeting Plasmodium ligands on mosquito salivary glands and midgut with a phage display peptide library.* Proc Natl Acad Sci U S A, 2001. **98**(23): p. 13278-81.

65. Ito, J., et al., *Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite.* Nature, 2002. **417**(6887): p. 452-5.

66. Moreira, L.A., et al., *Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes.* J Biol Chem, 2002. **277**(43): p. 40839-43.

67. Yoshida, S., et al., *Hemolytic C-type lectin CEL-III from sea cucumber expressed in transgenic mosquitoes impairs malaria parasite development.* PLoS Pathog, 2007. **3**(12): p. e192.

68. Dong, Y., et al., *Engineered anopheles immunity to Plasmodium infection.* PLoS Pathog, 2011. **7**(12): p. e1002458.

69. Corby-Harris, V., et al., *Activation of Akt signaling reduces the prevalence and intensity of malaria parasite infection and lifespan in Anopheles stephensi mosquitoes.* PLoS Pathog, 2010. **6**(7): p. e1001003.

70. Isaacs, A.T., et al., *Engineered resistance to Plasmodium falciparum development in transgenic Anopheles stephensi.* PLoS Pathog, 2011. **7**(4): p. e1002017.

71. Abraham, E.G., et al., *Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with AgAper1 regulatory elements.* Insect Mol Biol, 2005. **14**(3): p. 271-9.

72. Dong, Y., M.L. Simoes, and G. Dimopoulos, *Versatile transgenic multistage effector-gene combinations for Plasmodium falciparum suppression in Anopheles.* Sci Adv, 2020. **6**(20): p. eaay5898.

73. Hammond, A.M., et al., *The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito.* PLoS Genet, 2017. **13**(10): p. e1007039.

74. Austin Burt, M.C., Andrea Crisanti, Abdoulaye Diabate & Jonathan K. Kayondo, *Gene drive to reduce malaria transmission in sub-Saharan Africa.* Journal of Responsible Innovation, 2018.

75. Tu, Z. and C. Coates, *Mosquito transposable elements.* Insect Biochem Mol Biol, 2004. **34**(7): p. 631-44.

76. Horn, C., et al., *piggyBac-based insertional mutagenesis and enhancer detection as a tool for functional insect genomics.* Genetics, 2003. **163**(2): p. 647-61.

77. Klinakis, A.G., et al., *Mobility assays confirm the broad host-range activity of the Minos transposable element and validate new transformation tools.* Insect Mol Biol, 2000. **9**(3): p. 269-75.

78. Spradling, A.C., et al., *The Berkeley Drosophila Genome Project gene disruption project: Single P-element insertions mutating 25% of vital Drosophila genes.* Genetics, 1999. **153**(1): p. 135-77.

79. Handler AM, O.B.D., *Transposable elements for insect transformation*, in *Comprehensive Molecular Insect Science*. 2005, Elsevier: Oxford, UK. p. 437–474.

80. Windbichler, N., et al., *A synthetic homing endonuclease-based gene drive system in the human malaria mosquito.* Nature, 2011. **473**(7346): p. 212-5.

81. Pazdernik, D.P.C.a.N.J., *Inherited Defects and Gene Therapy*, in *Biotechnology Applying the Genetic Revolution*. 2015. p. 523-564.

82. Jinek, M., et al., *A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity.* Science, 2012. **337**(6096): p. 816-21.

83. Esvelt, K.M., et al., *Concerning RNA-guided gene drives for the alteration of wild populations.* Elife, 2014. **3**.

84. DiCarlo, J.E., et al., *Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems.* Nucleic Acids Res, 2013. **41**(7): p. 4336-43.

85. Gantz, V.M., et al., *Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi.* Proc Natl Acad Sci U S A, 2015. **112**(49): p. E6736-43.

86. Gantz, V.M. and E. Bier, *Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations.* Science, 2015. **348**(6233): p. 442-4.

87. Zug, R. and P. Hammerstein, *Still a host of hosts for Wolbachia: analysis of recent data suggests that 40% of terrestrial arthropod species are infected.* PLoS One, 2012. **7**(6): p. e38544.

88. Hilgenboecker, K., et al., *How many species are infected with Wolbachia? - a statistical analysis of current data.* Fems Microbiology Letters, 2008. **281**(2): p. 215-220.

89. Weinert, L.A., et al., *The incidence of bacterial endosymbionts in terrestrial arthropods.* Proceedings of the Royal Society B-Biological Sciences, 2015. **282**(1807).

90. Stouthamer, R., J.A.J. Breeuwer, and G.D.D. Hurst, *Wolbachia pipientis: Microbial manipulator of arthropod reproduction.* Annual Review of Microbiology, 1999. **53**: p. 71-102.

91. Werren, J.H., *Biology of Wolbachia.* Annual Review of Entomology, 1997. **42**: p. 587-609.

92. Bourtzis, K., et al., *Rescuing Wolbachia have been overlooked ...* Nature, 1998. **391**(6670): p. 852-853.

93. Bourtzis, K., Braig, H. & Karr, T. L., in *Insect symbiosis*, K.B.a.T.A. Miller., Editor. 2003, CRC: Boca Raton, FL. p. 217-246.

94. Leftwich, P.T., et al., *Recent advances in threshold-dependent gene drives for mosquitoes.* Biochem Soc Trans, 2018. **46**(5): p. 1203-1212.

95. Buchman, A., et al., *Synthetically engineered Medea gene drive system in the worldwide crop pest Drosophila suzukii.* Proc Natl Acad Sci U S A, 2018. **115**(18): p. 4725-4730.

96. Hillyer, J.F., *Mosquito immunity.* Adv Exp Med Biol, 2010. **708**: p. 218-38.

97. Boehm, T., et al., *VLR-based adaptive immunity.* Annu Rev Immunol, 2012. **30**: p. 203-20.

98. Kurtz, J. and S.A. Armitage, *Alternative adaptive immunity in invertebrates.* Trends Immunol, 2006. **27**(11): p. 493-6.

99. Aliota, M.T., et al., *Mosquito transcriptome changes and filarial worm resistance in Armigeres subalbatus.* BMC Genomics, 2007. **8**: p. 463.

100. Bartholomay, L.C., et al., *Description of the transcriptomes of immune response-activated hemocytes from the mosquito vectors Aedes aegypti and Armigeres subalbatus.* Infect Immun, 2004. **72**(7): p. 4114-26.

101. Bartholomay, L.C., et al., *Profiling infection responses in the haemocytes of the mosquito, Aedes aegypti.* Insect Mol Biol, 2007. **16**(6): p. 761-76.

102. Baton, L.A., et al., *Genome-wide transcriptomic profiling of Anopheles gambiae hemocytes reveals pathogen-specific signatures upon bacterial challenge and Plasmodium berghei infection.* BMC Genomics, 2009. **10**: p. 257.

103. Choi, Y.J., et al., *Tissue-enriched expression profiles in Aedes aegypti identify hemocyte-specific transcriptome responses to infection.* Insect Biochem Mol Biol, 2012. **42**(10): p. 729-38.

104. Pinto, S.B., et al., *Discovery of Plasmodium modulators by genome-wide analysis of circulating hemocytes in Anopheles gambiae.* Proc Natl Acad Sci U S A, 2009. **106**(50): p. 21270-5.

105. Hillyer, J.F. and M.R. Strand, *Mosquito hemocyte-mediated immune responses.* Curr Opin Insect Sci, 2014. **3**: p. 14-21.

106. Sheehan, G., et al., *Innate humoral immune defences in mammals and insects: The same, with differences ?* Virulence, 2018. **9**(1): p. 1625-1639.

107. Faye H. Rodgers, M.G., George K. Christophides, *The Mosquito Immune System and Its Interactions With the Microbiota: Implications for Disease Transmission*, in *Arthropod Vector: Controller of Disease Transmission*, S.A.G.D. Stephen K. Wikel, Editor. 2017.

108. Medzhitov, R. and C.A. Janeway, Jr., *Innate immunity: the virtues of a nonclonal system of recognition.* Cell, 1997. **91**(3): p. 295-8.

109. Medzhitov, R. and C.A. Janeway, Jr., *Decoding the patterns of self and nonself by the innate immune system.* Science, 2002. **296**(5566): p. 298-300.

110. Royet, J., *Infectious non-self recognition in invertebrates: lessons from Drosophila and other insect models.* Mol Immunol, 2004. **41**(11): p. 1063-75.

111. Gobert, V., et al., *Dual activation of the Drosophila toll pathway by two pattern recognition receptors.* Science, 2003. **302**(5653): p. 2126-30.

112. Hoffmann, J.A., *The immune response of Drosophila.* Nature, 2003. **426**(6962): p. 33-8.

113. Wilson, R., C. Chen, and N.A. Ratcliffe, *Innate immunity in insects: the role of multiple, endogenous serum lectins in the recognition of foreign invaders in the cockroach, Blaberus discoidalis.* J Immunol, 1999. **162**(3): p. 1590-6.

114. Yu, X.Q., et al., *Pattern recognition proteins in Manduca sexta plasma.* Insect Biochem Mol Biol, 2002. **32**(10): p. 1287-93.

115. Choe, K.M., et al., *Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila.* Science, 2002. **296**(5566): p. 359-62.

116. Levashina, E.A., et al., *Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, Anopheles gambiae.* Cell, 2001. **104**(5): p. 709-18.

117. *Insect Infection and Immunity Evolution, Ecology, and Mechanisms*. RES Symposia Series Vol. 25. 2009: Oxford University Press.

118. Yoshida, H., K. Kinoshita, and M. Ashida, *Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, Bombyx mori.* J Biol Chem, 1996. **271**(23): p. 13854-60.

119. Kang, D., et al., *A peptidoglycan recognition protein in innate immunity conserved from insects to humans.* Proc Natl Acad Sci U S A, 1998. **95**(17): p. 10078-82.

120. Werner, T., et al., *A family of peptidoglycan recognition proteins in the fruit fly Drosophila melanogaster.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13772-7.

121. Royet, J. and R. Dziarski, *Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences.* Nat Rev Microbiol, 2007. **5**(4): p. 264-77.

122. Dziarski, R., *Peptidoglycan recognition proteins (PGRPs).* Mol Immunol, 2004. **40**(12): p. 877-86.

123. Christophides, G.K., et al., *Immunity-related genes and gene families in Anopheles gambiae.* Science, 2002. **298**(5591): p. 159-65.

124. Meister, S., et al., *Anopheles gambiae PGRPLC-mediated defense against bacteria modulates infections with malaria parasites.* PLoS Pathog, 2009. **5**(8): p. e1000542.

125. Dimopoulos, G., et al., *Genome expression analysis of Anopheles gambiae: responses to injury, bacterial challenge, and malaria infection.* Proc Natl Acad Sci U S A, 2002. **99**(13): p. 8814-9.

126. Gendrin, M., et al., *The Peptidoglycan Recognition Proteins PGRPLA and PGRPLB Regulate Anopheles Immunity to Bacteria and Affect Infection by Plasmodium.* J Innate Immun, 2017. **9**(4): p. 333-342.

127. Dong, Y., F. Manfredini, and G. Dimopoulos, *Implication of the mosquito midgut microbiota in the defense against malaria parasites.* PLoS Pathog, 2009. **5**(5): p. e1000423.

128. Song, X., et al., *PGRP-LD mediates A. stephensi vector competency by regulating homeostasis of microbiota-induced peritrophic matrix synthesis.* PLoS Pathog, 2018. **14**(2): p. e1006899.

129. Waterhouse, R.M., et al., *Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes.* Science, 2007. **316**(5832): p. 1738-43.

130. Ochiai, M. and M. Ashida, *Purification of a beta-1,3-glucan recognition protein in the prophenoloxidase activating system from hemolymph of the silkworm, Bombyx mori.* J Biol Chem, 1988. **263**(24): p. 12056-62.

131. Ochiai, M. and M. Ashida, *A pattern-recognition protein for beta-1,3-glucan. The binding domain and the cDNA cloning of beta-1,3-glucan recognition protein from the silkworm, Bombyx mori.* J Biol Chem, 2000. **275**(7): p. 4995-5002.

132. Ochiai, M., T. Niki, and M. Ashida, *Immunocytochemical localization of beta-1,3-glucan recognition protein in the silkworm, Bombyx mori.* Cell Tissue Res, 1992. **268**(3): p. 431-7.

133. Warr, E., et al., *The Gram-negative bacteria-binding protein gene family: its role in the innate immune system of Anopheles gambiae and in anti-Plasmodium defence.* Insect Mol Biol, 2008. **17**(1): p. 39-51.

134. Gokudan, S., et al., *Horseshoe crab acetyl group-recognizing lectins involved in innate immunity are structurally related to fibrinogen.* Proc Natl Acad Sci U S A, 1999. **96**(18): p. 10086-91.

135. Wang, X., Q. Zhao, and B.M. Christensen, *Identification and characterization of the fibrinogen-like domain of fibrinogen-related proteins in the mosquito, Anopheles gambiae, and the fruitfly, Drosophila melanogaster, genomes.* BMC Genomics, 2005. **6**: p. 114.

136. Adema, C.M., et al., *A family of fibrinogen-related proteins that precipitates parasite-derived molecules is produced by an invertebrate after infection.* Proc Natl Acad Sci U S A, 1997. **94**(16): p. 8691-6.

137. Zhang, S.M. and E.S. Loker, *The FREP gene family in the snail Biomphalaria glabrata: additional members, and evidence consistent with alternative splicing and FREP retrosequences. Fibrinogen-related proteins.* Dev Comp Immunol, 2003. **27**(3): p. 175-87.

138. Dong, Y. and G. Dimopoulos, *Anopheles fibrinogen-related proteins provide expanded pattern recognition capacity against bacteria and malaria parasites.* J Biol Chem, 2009. **284**(15): p. 9835-44.

139. Dong, Y., et al., *Anopheles gambiae immune responses to human and rodent Plasmodium parasite species.* PLoS Pathog, 2006. **2**(6): p. e52.

140. Simoes, M.L., et al., *The Anopheles FBN9 immune factor mediates Plasmodium species-specific defense through transgenic fat body expression.* Dev Comp Immunol, 2017. **67**: p. 257-265.

141. Garver, L.S., Z. Xi, and G. Dimopoulos, *Immunoglobulin superfamily members play an important role in the mosquito immune system.* Dev Comp Immunol, 2008. **32**(5): p. 519-31.

142. Dong, Y., H.E. Taylor, and G. Dimopoulos, *AgDscam, a hypervariable immunoglobulin domain-containing receptor of the Anopheles gambiae innate immune system.* PLoS Biol, 2006. **4**(7): p. e229.

143. Dong, Y., et al., *Anopheles NF-kappaB-regulated splicing factors direct pathogen-specific repertoires of the hypervariable pattern recognition receptor AgDscam.* Cell Host Microbe, 2012. **12**(4): p. 521-30.

144. Smith, P.H., et al., *Alternative splicing of the Anopheles gambiae Dscam gene in diverse Plasmodium falciparum infections.* Malar J, 2011. **10**: p. 156.

145. Drickamer, K. and M.E. Taylor, *Biology of animal lectins.* Annu Rev Cell Biol, 1993. **9**: p. 237-64.

146. Xia, X., et al., *Insect C-type lectins in innate immunity.* Dev Comp Immunol, 2018. **83**: p. 70-79.

147. Gerardo, N.M., et al., *Immunity and other defenses in pea aphids, Acyrthosiphon pisum.* Genome Biol, 2010. **11**(2): p. R21.

148. Rao, X.J., et al., *Identification of C-type lectin-domain proteins (CTLDPs) in silkworm Bombyx mori.* Dev Comp Immunol, 2015. **53**(2): p. 328-38.

149. Rao, X.J., et al., *Structural features, evolutionary relationships, and transcriptional regulation of C-type lectin-domain proteins in Manduca sexta.* Insect Biochem Mol Biol, 2015. **62**: p. 75-85.

150. Xia, X., et al., *Genome-wide characterization and expression profiling of immune genes in the diamondback moth, Plutella xylostella (L.).* Sci Rep, 2015. **5**: p. 9877.

151. Pang, X., et al., *Mosquito C-type lectins maintain gut microbiome homeostasis.* Nat Microbiol, 2016. **1**.

152. Cheng, G., et al., *A C-type lectin collaborates with a CD45 phosphatase homolog to facilitate West Nile virus infection of mosquitoes.* Cell, 2010. **142**(5): p. 714-25.

153. Liu, Y., et al., *Transmission-blocking antibodies against mosquito C-type lectins for dengue prevention.* PLoS Pathog, 2014. **10**(2): p. e1003931.

154. Ling, E., J. Ao, and X.Q. Yu, *Nuclear translocation of immulectin-3 stimulates hemocyte proliferation.* Mol Immunol, 2008. **45**(9): p. 2598-606.

155. Schnitger, A.K., et al., *Two C-type lectins cooperate to defend Anopheles gambiae against Gram-negative bacteria.* J Biol Chem, 2009. **284**(26): p. 17616-24.

156. Osta, M.A., G.K. Christophides, and F.C. Kafatos, *Effects of mosquito genes on Plasmodium development.* Science, 2004. **303**(5666): p. 2030-2.

157. Warr, E., et al., *Anopheles gambiae immune responses to Sephadex beads: involvement of anti-Plasmodium factors in regulating melanization.* Insect Biochem Mol Biol, 2006. **36**(10): p. 769-78.

158. Cohuet, A., et al., *Anopheles and Plasmodium: from laboratory models to natural systems in the field.* EMBO Rep, 2006. **7**(12): p. 1285-9.

159. Simoes, M.L., et al., *Immune Regulation of Plasmodium Is Anopheles Species Specific and Infection Intensity Dependent.* mBio, 2017. **8**(5).

160. Nusslein-Volhard, C. and E. Wieschaus, *Mutations affecting segment number and polarity in Drosophila.* Nature, 1980. **287**(5785): p. 795-801.

161. Ligoxygakis, P., et al., *Activation of Drosophila Toll during fungal infection by a blood serine protease.* Science, 2002. **297**(5578): p. 114-6.

162. Lemaitre, B., et al., *Functional analysis and regulation of nuclear import of dorsal during the immune response in Drosophila.* EMBO J, 1995. **14**(3): p. 536-45.

163. Lemaitre, B., et al., *The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults.* Cell, 1996. **86**(6): p. 973-83.

164. Levashina, E.A., et al., *Constitutive activation of toll-mediated antifungal defense in serpin-deficient Drosophila.* Science, 1999. **285**(5435): p. 1917-9.

165. Horng, T. and R. Medzhitov, *Drosophila MyD88 is an adapter in the Toll signaling pathway.* Proc Natl Acad Sci U S A, 2001. **98**(22): p. 12654-8.

166. Lemaitre, B. and J. Hoffmann, *The host defense of Drosophila melanogaster.* Annu Rev Immunol, 2007. **25**: p. 697-743.

167. Valanne, S., J.H. Wang, and M. Ramet, *The Drosophila Toll signaling pathway.* J Immunol, 2011. **186**(2): p. 649-56.

168. Leulier, F., et al., *The Drosophila immune system detects bacteria through specific peptidoglycan recognition.* Nat Immunol, 2003. **4**(5): p. 478-84.

169. Buchon, N., et al., *A single modular serine protease integrates signals from pattern-recognition receptors upstream of the Drosophila Toll pathway.* Proc Natl Acad Sci U S A, 2009. **106**(30): p. 12442-7.

170. El Chamy, L., et al., *Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of Toll.* Nat Immunol, 2008. **9**(10): p. 1165-70.

171. Gottar, M., et al., *Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors.* Cell, 2006. **127**(7): p. 1425-37.

172. Pili-Floury, S., et al., *In vivo RNA interference analysis reveals an unexpected role for GNBP1 in the defense against Gram-positive bacterial infection in Drosophila adults.* J Biol Chem, 2004. **279**(13): p. 12848-53.

173. Imler, J.L. and P. Bulet, *Antimicrobial peptides in Drosophila: structures, activities and gene regulation.* Chem Immunol Allergy, 2005. **86**: p. 1-21.

174. Shen, B., et al., *Physical and functional interactions between Drosophila TRAF2 and Pelle kinase contribute to Dorsal activation.* Proc Natl Acad Sci U S A, 2001. **98**(15): p. 8596-601.

175. Fehlbaum, P., et al., *Insect immunity. Septic injury of Drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides.* J Biol Chem, 1994. **269**(52): p. 33159-63.

176. Levashina, E.A., et al., *Metchnikowin, a novel immune-inducible proline-rich peptide from Drosophila with antibacterial and antifungal properties.* Eur J Biochem, 1995. **233**(2): p. 694-700.

177. Nicolas, E., et al., *In vivo regulation of the IkappaB homologue cactus during the immune response of Drosophila.* J Biol Chem, 1998. **273**(17): p. 10463-9.

178. Qiu, P., P.C. Pan, and S. Govind, *A role for the Drosophila Toll/Cactus pathway in larval hematopoiesis.* Development, 1998. **125**(10): p. 1909-20.

179. Carton, Y. and A.J. Nappi, *Immunogenetic aspects of the cellular immune response of Drosophilia against parasitoids.* Immunogenetics, 2001. **52**(3-4): p. 157-64.

180. Bian, G., et al., *Transgenic alteration of Toll immune pathway in the female mosquito Aedes aegypti.* Proc Natl Acad Sci U S A, 2005. **102**(38): p. 13568-73.

181. Ramirez, J.L. and G. Dimopoulos, *The Toll immune signaling pathway control conserved anti-dengue defenses across diverse Ae. aegypti strains and against multiple dengue virus serotypes.* Dev Comp Immunol, 2010. **34**(6): p. 625-9.

182. Vizioli, J., et al., *Gambicin: a novel immune responsive antimicrobial peptide from the malaria vector Anopheles gambiae.* Proc Natl Acad Sci U S A, 2001. **98**(22): p. 12630-5.

183. Frolet, C., et al., *Boosting NF-kappaB-dependent basal immunity of Anopheles gambiae aborts development of Plasmodium berghei.* Immunity, 2006. **25**(4): p. 677-85.

184. Garver, L.S., Y. Dong, and G. Dimopoulos, *Caspar controls resistance to Plasmodium falciparum in diverse anopheline species.* PLoS Pathog, 2009. **5**(3): p. e1000335.

185. Ramirez, J.L., et al., *The role of hemocytes in Anopheles gambiae antiplasmodial immunity.* J Innate Immun, 2014. **6**(2): p. 119-28.

186. Shin, S.W., et al., *REL1, a homologue of Drosophila dorsal, regulates toll antifungal immune pathway in the female mosquito Aedes aegypti.* J Biol Chem, 2005. **280**(16): p. 16499-507.

187. Shin, S.W., G. Bian, and A.S. Raikhel, *A toll receptor and a cytokine, Toll5A and Spz1C, are involved in toll antifungal immune signaling in the mosquito Aedes aegypti.* J Biol Chem, 2006. **281**(51): p. 39388-95.

188. Xi, Z., J.L. Ramirez, and G. Dimopoulos, *The Aedes aegypti toll pathway controls dengue virus infection.* PLoS Pathog, 2008. **4**(7): p. e1000098.

189. Luplertlop, N., et al., *Induction of a peptide with activity against a broad spectrum of pathogens in the Aedes aegypti salivary gland, following Infection with Dengue Virus.* PLoS Pathog, 2011. **7**(1): p. e1001252.

190. Sim, S. and G. Dimopoulos, *Dengue virus inhibits immune responses in Aedes aegypti cells.* PLoS One, 2010. **5**(5): p. e10678.

191. Dong, Y., et al., *The entomopathogenic fungus Beauveria bassiana activate toll and JAK-STAT pathway-controlled effector genes and anti-dengue activity in Aedes aegypti.* Insect Biochem Mol Biol, 2012. **42**(2): p. 126-32.

192. Pan, X., et al., *Wolbachia induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito Aedes aegypti.* Proc Natl Acad Sci U S A, 2012. **109**(1): p. E23-31.

193. Anglero-Rodriguez, Y.I., et al., *Aedes aegypti Molecular Responses to Zika Virus: Modulation of Infection by the Toll and Jak/Stat Immune Pathways and Virus Host Factors.* Front Microbiol, 2017. **8**: p. 2050.

194. Barletta, A.B., et al., *Emerging role of lipid droplets in Aedes aegypti immune response against bacteria and Dengue virus.* Sci Rep, 2016. **6**: p. 19928.

195. Ramet, M., et al., *Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli.* Nature, 2002. **416**(6881): p. 644-8.

196. Gottar, M., et al., *The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein.* Nature, 2002. **416**(6881): p. 640-4.

197. Choe, K.M., H. Lee, and K.V. Anderson, *Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor.* Proc Natl Acad Sci U S A, 2005. **102**(4): p. 1122-6.

198. Kaneko, T., et al., *Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway.* Immunity, 2004. **20**(5): p. 637-49.

199. Maillet, F., et al., *The Drosophila peptidoglycan recognition protein PGRP-LF blocks PGRP-LC and IMD/JNK pathway activation.* Cell Host Microbe, 2008. **3**(5): p. 293-303.

200. Basbous, N., et al., *The Drosophila peptidoglycan-recognition protein LF interacts with peptidoglycan-recognition protein LC to downregulate the Imd pathway.* EMBO Rep, 2011. **12**(4): p. 327-33.

201. Zaidman-Remy, A., et al., *The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection.* Immunity, 2006. **24**(4): p. 463-73.

202. Paredes, J.C., et al., *Negative regulation by amidase PGRPs shapes the Drosophila antibacterial response and protects the fly from innocuous infection.* Immunity, 2011. **35**(5): p. 770-9.

203. Bischoff, V., et al., *Downregulation of the Drosophila immune response by peptidoglycan-recognition proteins SC1 and SC2.* PLoS Pathog, 2006. **2**(2): p. e14.

204. Takehana, A., et al., *Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in Drosophila larvae.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13705-10.

205. Takehana, A., et al., *Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in Drosophila immunity.* EMBO J, 2004. **23**(23): p. 4690-700.

206. Kaneko, T., et al., *PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan.* Nat Immunol, 2006. **7**(7): p. 715-23.

207. Gendrin, M., et al., *Functional analysis of PGRP-LA in Drosophila immunity.* PLoS One, 2013. **8**(7): p. e69742.

208. Lemaitre, B., et al., *A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense.* Proc Natl Acad Sci U S A, 1995. **92**(21): p. 9465-9.

209. Georgel, P., et al., *Drosophila immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis.* Dev Cell, 2001. **1**(4): p. 503-14.

210. Leulier, F., et al., *The Drosophila caspase Dredd is required to resist gram-negative bacterial infection.* EMBO Rep, 2000. **1**(4): p. 353-8.

211. Leulier, F., et al., *Inducible expression of double-stranded RNA reveals a role for dFADD in the regulation of the antibacterial response in Drosophila adults.* Curr Biol, 2002. **12**(12): p. 996-1000.

212. Meinander, A., et al., *Ubiquitylation of the initiator caspase DREDD is required for innate immune signalling.* EMBO J, 2012. **31**(12): p. 2770-83.

213. Zhou, R., et al., *The role of ubiquitination in Drosophila innate immunity.* J Biol Chem, 2005. **280**(40): p. 34048-55.

214. Paquette, N., et al., *Caspase-mediated cleavage, IAP binding, and ubiquitination: linking three mechanisms crucial for Drosophila NF-kappaB signaling.* Mol Cell, 2010. **37**(2): p. 172-82.

215. Lu, Y., L.P. Wu, and K.V. Anderson, *The antibacterial arm of the drosophila innate immune response requires an IkappaB kinase.* Genes Dev, 2001. **15**(1): p. 104-10.

216. Rutschmann, S., et al., *Role of Drosophila IKK gamma in a toll-independent antibacterial immune response.* Nat Immunol, 2000. **1**(4): p. 342-7.

217. Silverman, N., et al., *A Drosophila IkappaB kinase complex required for Relish cleavage and antibacterial immunity.* Genes Dev, 2000. **14**(19): p. 2461-71.

218. Kleino, A., et al., *Inhibitor of apoptosis 2 and TAK1-binding protein are components of the Drosophila Imd pathway.* EMBO J, 2005. **24**(19): p. 3423-34.

219. Erturk-Hasdemir, D., et al., *Two roles for the Drosophila IKK complex in the activation of Relish and the induction of antimicrobial peptide genes.* Proc Natl Acad Sci U S A, 2009. **106**(24): p. 9779-84.

220. Stoven, S., et al., *Caspase-mediated processing of the Drosophila NF-kappaB factor Relish.* Proc Natl Acad Sci U S A, 2003. **100**(10): p. 5991-6.

221. Stoven, S., et al., *Activation of the Drosophila NF-kappaB factor Relish by rapid endoproteolytic cleavage.* EMBO Rep, 2000. **1**(4): p. 347-52.

222. Goto, A., et al., *Akirins are highly conserved nuclear proteins required for NF-kappaB-dependent gene expression in drosophila and mice.* Nat Immunol, 2008. **9**(1): p. 97-104.

223. Kleino, A., et al., *Pirk is a negative regulator of the Drosophila Imd pathway.* J Immunol, 2008. **180**(8): p. 5413-22.

224. Kallio, J., et al., *Functional analysis of immune response genes in Drosophila identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells.* Microbes Infect, 2005. **7**(5-6): p. 811-9.

225. Kim, M., et al., *Caspar, a suppressor of antibacterial immunity in Drosophila.* Proc Natl Acad Sci U S A, 2006. **103**(44): p. 16358-63.

226. Foley, E. and P.H. O'Farrell, *Functional dissection of an innate immune response by a genome-wide RNAi screen.* PLoS Biol, 2004. **2**(8): p. E203.

227. Delaney, J.R., et al., *Cooperative control of Drosophila immune responses by the JNK and NF-kappaB signaling pathways.* EMBO J, 2006. **25**(13): p. 3068-77.

228. Silverman, N., et al., *Immune activation of NF-kappaB and JNK requires Drosophila TAK1.* J Biol Chem, 2003. **278**(49): p. 48928-34.

229. Valanne, S., et al., *Iap2 is required for a sustained response in the Drosophila Imd pathway.* Dev Comp Immunol, 2007. **31**(10): p. 991-1001.

230. Meister, S., et al., *Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito Anopheles gambiae.* Proc Natl Acad Sci U S A, 2005. **102**(32): p. 11420-5.

231. Garver, L.S., et al., *Anopheles Imd pathway factors and effectors in infection intensity-dependent anti-Plasmodium action.* PLoS Pathog, 2012. **8**(6): p. e1002737.

232. Barletta, A.B., et al., *Microbiota activates IMD pathway and limits Sindbis infection in Aedes aegypti.* Parasit Vectors, 2017. **10**(1): p. 103.

233. Myllymaki, H. and M. Ramet, *JAK/STAT pathway in Drosophila immunity.* Scand J Immunol, 2014. **79**(6): p. 377-85.

234. Harrison, D.A., et al., *Drosophila unpaired encodes a secreted protein that activates the JAK signaling pathway.* Genes Dev, 1998. **12**(20): p. 3252-63.

235. Gilbert, M.M., et al., *A novel functional activator of the Drosophila JAK/STAT pathway, unpaired2, is revealed by an in vivo reporter of pathway activation.* Mech Dev, 2005. **122**(7-8): p. 939-48.

236. Hombria, J.C., et al., *Characterisation of Upd2, a Drosophila JAK/STAT pathway ligand.* Dev Biol, 2005. **288**(2): p. 420-33.

237. Agaisse, H., et al., *Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury.* Dev Cell, 2003. **5**(3): p. 441-50.

238. Wright, V.M., et al., *Differential activities of the Drosophila JAK/STAT pathway ligands Upd, Upd2 and Upd3.* Cell Signal, 2011. **23**(5): p. 920-7.

239. Brown, S., N. Hu, and J.C. Hombria, *Identification of the first invertebrate interleukin JAK/STAT receptor, the Drosophila gene domeless.* Curr Biol, 2001. **11**(21): p. 1700-5.

240. Brown, S., N. Hu, and J.C. Hombria, *Novel level of signalling control in the JAK/STAT pathway revealed by in situ visualisation of protein-protein interaction during Drosophila development.* Development, 2003. **130**(14): p. 3077-84.

241. Chen, H.W., et al., *mom identifies a receptor for the Drosophila JAK/STAT signal transduction pathway and encodes a protein distantly related to the mammalian cytokine receptor family.* Genes Dev, 2002. **16**(3): p. 388-98.

242. Pastor-Pareja, J.C., M. Wu, and T. Xu, *An innate immune response of blood cells to tumors and tissue damage in Drosophila.* Dis Model Mech, 2008. **1**(2-3): p. 144-54; discussion 153.

243. Kemp, C., et al., *Broad RNA interference-mediated antiviral immunity and virus-specific inducible responses in Drosophila.* J Immunol, 2013. **190**(2): p. 650-8.

244. Binari, R. and N. Perrimon, *Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila.* Genes Dev, 1994. **8**(3): p. 300-12.

245. Hou, X.S., M.B. Melnick, and N. Perrimon, *Marelle acts downstream of the Drosophila HOP/JAK kinase and encodes a protein similar to the mammalian STATs.* Cell, 1996. **84**(3): p. 411-9.

246. Yan, R., et al., *Identification of a Stat gene that functions in Drosophila development.* Cell, 1996. **84**(3): p. 421-30.

247. Rawlings, J.S., et al., *Two Drosophila suppressors of cytokine signaling (SOCS) differentially regulate JAK and EGFR pathway activities.* BMC Cell Biol, 2004. **5**(1): p. 38.

248. Stec, W.J. and M.P. Zeidler, *Drosophila SOCS Proteins.* J Signal Transduct, 2011. **2011**: p. 894510.

249. Callus, B.A. and B. Mathey-Prevot, *SOCS36E, a novel Drosophila SOCS protein, suppresses JAK/STAT and EGF-R signalling in the imaginal wing disc.* Oncogene, 2002. **21**(31): p. 4812-21.

250. Karsten, P., S. Hader, and M.P. Zeidler, *Cloning and expression of Drosophila SOCS36E and its potential regulation by the JAK/STAT pathway.* Mech Dev, 2002. **117**(1-2): p. 343-6.

251. Baeg, G.H., R. Zhou, and N. Perrimon, *Genome-wide RNAi analysis of JAK/STAT signaling components in Drosophila.* Genes Dev, 2005. **19**(16): p. 1861-70.

252. Muller, P., et al., *Identification of JAK/STAT signalling components by genome-wide RNA interference.* Nature, 2005. **436**(7052): p. 871-5.

253. Gronholm, J., et al., *Not4 enhances JAK/STAT pathway-dependent gene expression in Drosophila and in human cells.* FASEB J, 2012. **26**(3): p. 1239-50.

254. Chung, C.D., et al., *Specific inhibition of Stat3 signal transduction by PIAS3.* Science, 1997. **278**(5344): p. 1803-5.

255. Betz, A., et al., *A Drosophila PIAS homologue negatively regulates stat92E.* Proc Natl Acad Sci U S A, 2001. **98**(17): p. 9563-8.

256. Gronholm, J., et al., *Sumoylation of Drosophila transcription factor STAT92E.* J Innate Immun, 2010. **2**(6): p. 618-24.

257. Arbouzova, N.I., E.A. Bach, and M.P. Zeidler, *Ken & barbie selectively regulates the expression of a subset of Jak/STAT pathway target genes.* Curr Biol, 2006. **16**(1): p. 80-8.

258. Harris, M.B., J. Mostecki, and P.B. Rothman, *Repression of an interleukin-4-responsive promoter requires cooperative BCL-6 function.* J Biol Chem, 2005. **280**(13): p. 13114-21.

259. Coste, F., et al., *Crystal structure of Diedel, a marker of the immune response of Drosophila melanogaster.* PLoS One, 2012. **7**(3): p. e33416.

260. Shuai, K. and B. Liu, *Regulation of JAK-STAT signalling in the immune system.* Nat Rev Immunol, 2003. **3**(11): p. 900-11.

261. Barillas-Mury, C., et al., *Anopheles gambiae Ag-STAT, a new insect member of the STAT family, is activated in response to bacterial infection.* EMBO J, 1999. **18**(4): p. 959-67.

262. Gupta, L., et al., *The STAT pathway mediates late-phase immunity against Plasmodium in the mosquito Anopheles gambiae.* Cell Host Microbe, 2009. **5**(5): p. 498-507.

263. Souza-Neto, J.A., S. Sim, and G. Dimopoulos, *An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense.* Proc Natl Acad Sci U S A, 2009. **106**(42): p. 17841-6.

264. Jupatanakul, N., et al., *Engineered Aedes aegypti JAK/STAT Pathway-Mediated Immunity to Dengue Virus.* PLoS Negl Trop Dis, 2017. **11**(1): p. e0005187.

265. Horton, A.A., et al., *The mitogen-activated protein kinome from Anopheles gambiae: identification, phylogeny and functional characterization of the ERK, JNK and p38 MAP kinases.* BMC Genomics, 2011. **12**: p. 574.

266. Stronach, B., *Dissecting JNK signaling, one KKKinase at a time.* Dev Dyn, 2005. **232**(3): p. 575-84.

267. Boutros, M., H. Agaisse, and N. Perrimon, *Sequential activation of signaling pathways during innate immune responses in Drosophila.* Dev Cell, 2002. **3**(5): p. 711-22.

268. Chen, W., M.A. White, and M.H. Cobb, *Stimulus-specific requirements for MAP3 kinases in activating the JNK pathway.* J Biol Chem, 2002. **277**(51): p. 49105-10.

269. Geuking, P., et al., *A non-redundant role for Drosophila Mkk4 and hemipterous/Mkk7 in TAK1-mediated activation of JNK.* PLoS One, 2009. **4**(11): p. e7709.

270. Glise, B., H. Bourbon, and S. Noselli, *hemipterous encodes a novel Drosophila MAP kinase kinase, required for epithelial cell sheet movement.* Cell, 1995. **83**(3): p. 451-61.

271. Sluss, H.K., et al., *A JNK signal transduction pathway that mediates morphogenesis and an immune response in Drosophila.* Genes Dev, 1996. **10**(21): p. 2745-58.

272. Kockel, L., J.G. Homsy, and D. Bohmann, *Drosophila AP-1: lessons from an invertebrate.* Oncogene, 2001. **20**(19): p. 2347-64.

273. Libert, S., et al., *Realized immune response is enhanced in long-lived puc and chico mutants but is unaffected by dietary restriction.* Mol Immunol, 2008. **45**(3): p. 810-7.

274. Martin-Blanco, E., et al., *puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila.* Genes Dev, 1998. **12**(4): p. 557-70.

275. Park, J.M., et al., *Targeting of TAK1 by the NF-kappa B protein Relish regulates the JNK-mediated immune response in Drosophila.* Genes Dev, 2004. **18**(5): p. 584-94.

276. Garver, L.S., G. de Almeida Oliveira, and C. Barillas-Mury, *The JNK pathway is a key mediator of Anopheles gambiae antiplasmodial immunity.* PLoS Pathog, 2013. **9**(9): p. e1003622.

277. Oliveira Gde, A., J. Lieberman, and C. Barillas-Mury, *Epithelial nitration by a peroxidase/NOX5 system mediates mosquito antiplasmodial immunity.* Science, 2012. **335**(6070): p. 856-9.

278. Blandin, S., et al., *Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae.* Cell, 2004. **116**(5): p. 661-70.

279. Jaramillo-Gutierrez, G., et al., *The Anopheles gambiae oxidation resistance 1 (OXR1) gene regulates expression of enzymes that detoxify reactive oxygen species.* PLoS One, 2010. **5**(6): p. e11168.

280. Elliott, N.A. and M.R. Volkert, *Stress induction and mitochondrial localization of Oxr1 proteins in yeast and humans.* Mol Cell Biol, 2004. **24**(8): p. 3180-7.

281. Brandt, S.M., et al., *Use of a Drosophila model to identify genes regulating Plasmodium growth in the mosquito.* Genetics, 2008. **180**(3): p. 1671-8.

282. Liu, T., et al., *Antiviral systems in vector mosquitoes.* Dev Comp Immunol, 2018. **83**: p. 34-43.

283. Williams, M. and R. Baxter, *The structure and function of thioester-containing proteins in arthropods.* Biophys Rev, 2014. **6**(3-4): p. 261-272.

284. Dodds, A.W. and S.K. Law, *The phylogeny and evolution of the thioester bond-containing proteins C3, C4 and alpha 2-macroglobulin.* Immunol Rev, 1998. **166**: p. 15-26.

285. Shokal, U. and I. Eleftherianos, *Evolution and Function of Thioester-Containing Proteins and the Complement System in the Innate Immune Response.* Front Immunol, 2017. **8**: p. 759.

286. Lagueux, M., et al., *Constitutive expression of a complement-like protein in toll and JAK gain-of-function mutants of Drosophila.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11427-32.

287. Stroschein-Stevenson, S.L., et al., *Identification of Drosophila gene products required for phagocytosis of Candida albicans.* PLoS Biol, 2006. **4**(1): p. e4.

288. Vierstraete, E., et al., *A proteomic approach for the analysis of instantly released wound and immune proteins in Drosophila melanogaster hemolymph.* Proc Natl Acad Sci U S A, 2004. **101**(2): p. 470-5.

289. Blandin, S.A., E. Marois, and E.A. Levashina, *Antimalarial responses in Anopheles gambiae: from a complement-like protein to a complement-like pathway.* Cell Host Microbe, 2008. **3**(6): p. 364-74.

290. Nonaka, M. and A. Kimura, *Genomic view of the evolution of the complement system.* Immunogenetics, 2006. **58**(9): p. 701-13.

291. Christophides, G.K., D. Vlachou, and F.C. Kafatos, *Comparative and functional genomics of the innate immune system in the malaria vector Anopheles gambiae.* Immunol Rev, 2004. **198**: p. 127-48.

292. Baxter, R.H., et al., *Structural basis for conserved complement factor-like function in the antimalarial protein TEP1.* Proc Natl Acad Sci U S A, 2007. **104**(28): p. 11615-20.

293. Fraiture, M., et al., *Two mosquito LRR proteins function as complement control factors in the TEP1-mediated killing of Plasmodium.* Cell Host Microbe, 2009. **5**(3): p. 273-84.

294. Povelones, M., et al., *Leucine-rich repeat protein complex activates mosquito complement in defense against Plasmodium parasites.* Science, 2009. **324**(5924): p. 258-61.

295. Povelones, M., et al., *Structure-function analysis of the Anopheles gambiae LRIM1/APL1C complex and its interaction with complement C3-like protein TEP1.* PLoS Pathog, 2011. **7**(4): p. e1002023.

296. Baxter, R.H., et al., *A heterodimeric complex of the LRR proteins LRIM1 and APL1C regulates complement-like immunity in Anopheles gambiae.* Proc Natl Acad Sci U S A, 2010. **107**(39): p. 16817-22.

297. Le, B.V., et al., *Molecular basis for genetic resistance of Anopheles gambiae to Plasmodium: structural analysis of TEP1 susceptible and resistant alleles.* PLoS Pathog, 2012. **8**(10): p. e1002958.

298. Blandin, S.A., et al., *Dissecting the genetic basis of resistance to malaria parasites in Anopheles gambiae.* Science, 2009. **326**(5949): p. 147-50.

299. Mitri, C., et al., *Fine pathogen discrimination within the APL1 gene family protects Anopheles gambiae against human and rodent malaria species.* PLoS Pathog, 2009. **5**(9): p. e1000576.

300. Povelones, M., et al., *The CLIP-domain serine protease homolog SPCLIP1 regulates complement recruitment to microbial surfaces in the malaria mosquito Anopheles gambiae.* PLoS Pathog, 2013. **9**(9): p. e1003623.

301. Yassine, H., et al., *A serine protease homolog negatively regulates TEP1 consumption in systemic infections of the malaria vector Anopheles gambiae.* J Innate Immun, 2014. **6**(6): p. 806-18.

302. Kamareddine, L., J. Nakhleh, and M.A. Osta, *Functional Interaction between Apolipophorins and Complement Regulate the Mosquito Immune Response to Systemic Infections.* J Innate Immun, 2016. **8**(3): p. 314-26.

303. White, B.J., et al., *Adaptive divergence between incipient species of Anopheles gambiae increases resistance to Plasmodium.* Proc Natl Acad Sci U S A, 2011. **108**(1): p. 244-9.

304. Collins, F.H., et al., *Genetic selection of a Plasmodium-refractory strain of the malaria vector Anopheles gambiae.* Science, 1986. **234**(4776): p. 607-10.

305. Molina-Cruz, A., et al., *Some strains of Plasmodium falciparum, a human malaria parasite, evade the complement-like system of Anopheles gambiae mosquitoes.* Proc Natl Acad Sci U S A, 2012. **109**(28): p. E1957-62.

306. Volohonsky, G., et al., *Transgenic Expression of the Anti-parasitic Factor TEP1 in the Malaria Mosquito Anopheles gambiae.* PLoS Pathog, 2017. **13**(1): p. e1006113.

307. Shiao, S.H., et al., *Fz2 and cdc42 mediate melanization and actin polymerization but are dispensable for Plasmodium killing in the mosquito midgut.* PLoS Pathog, 2006. **2**(12): p. e133.

308. Molina-Cruz, A., et al., *The human malaria parasite Pfs47 gene mediates evasion of the mosquito immune system.* Science, 2013. **340**(6135): p. 984-7.

309. Eldering, M., et al., *Variation in susceptibility of African Plasmodium falciparum malaria parasites to TEP1 mediated killing in Anopheles gambiae mosquitoes.* Sci Rep, 2016. **6**: p. 20440.

310. Moita, L.F., et al., *In vivo identification of novel regulators and conserved pathways of phagocytosis in A. gambiae.* Immunity, 2005. **23**(1): p. 65-73.

311. Yassine, H., L. Kamareddine, and M.A. Osta, *The mosquito melanization response is implicated in defense against the entomopathogenic fungus Beauveria bassiana.* PLoS Pathog, 2012. **8**(11): p. e1003029.

312. Bulet, P., et al., *Antimicrobial peptides in insects; structure and function.* Dev Comp Immunol, 1999. **23**(4-5): p. 329-44.

313. Vizioli, J., et al., *Cloning and analysis of a cecropin gene from the malaria vector mosquito, Anopheles gambiae.* Insect Mol Biol, 2000. **9**(1): p. 75-84.

314. Kim, W., et al., *Ectopic expression of a cecropin transgene in the human malaria vector mosquito Anopheles gambiae (Diptera: Culicidae): effects on susceptibility to Plasmodium.* J Med Entomol, 2004. **41**(3): p. 447-55.

315. Gwadz, R.W., et al., *Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes.* Infect Immun, 1989. **57**(9): p. 2628-33.

316. Vizioli, J., et al., *The defensin peptide of the malaria vector mosquito Anopheles gambiae: antimicrobial activities and expression in adult mosquitoes.* Insect Biochem Mol Biol, 2001. **31**(3): p. 241-8.

317. Dimopoulos, G., et al., *Malaria infection of the mosquito Anopheles gambiae activates immune-responsive genes during critical transition stages of the parasite life cycle.* Embo J, 1998. **17**(21): p. 6115-23.

318. Richman, A.M., et al., *Plasmodium activates the innate immune response of Anopheles gambiae mosquitoes.* EMBO J, 1997. **16**(20): p. 6114-9.

319. Dimopoulos, G., et al., *Molecular immune responses of the mosquito Anopheles gambiae to bacteria and malaria parasites.* Proc Natl Acad Sci U S A, 1997. **94**(21): p. 11508-13.

320. Richman, A.M., et al., *Inducible immune factors of the vector mosquito Anopheles gambiae: biochemical purification of a defensin antibacterial peptide and molecular cloning of preprodefensin cDNA.* Insect Mol Biol, 1996. **5**(3): p. 203-10.

321. Blandin, S., et al., *Reverse genetics in the mosquito Anopheles gambiae: targeted disruption of the Defensin gene.* EMBO Rep, 2002. **3**(9): p. 852-6.

322. Arrighi, R.B., et al., *The immunogenic properties of protozoan glycosylphosphatidylinositols in the mosquito Anopheles gambiae.* Dev Comp Immunol, 2009. **33**(2): p. 216-23.

323. Luckhart, S. and R. Rosenberg, *Gene structure and polymorphism of an invertebrate nitric oxide synthase gene.* Gene, 1999. **232**(1): p. 25-34.

324. Luckhart, S. and K. Li, *Transcriptional complexity of the Anopheles stephensi nitric oxide synthase gene.* Insect Biochem Mol Biol, 2001. **31**(3): p. 249-56.

325. Luckhart, S., et al., *The mosquito Anopheles stephensi limits malaria parasite development with inducible synthesis of nitric oxide.* Proc Natl Acad Sci U S A, 1998. **95**(10): p. 5700-5.

326. Crampton, A. and S. Luckhart, *The role of As60A, a TGF-beta homolog, in Anopheles stephensi innate immunity and defense against Plasmodium infection.* Infect Genet Evol, 2001. **1**(2): p. 131-41.

327. Luckhart, S., et al., *Mammalian transforming growth factor beta1 activated after ingestion by Anopheles stephensi modulates mosquito immunity.* Infect Immun, 2003. **71**(6): p. 3000-9.

328. Han, Y.S., et al., *Molecular interactions between Anopheles stephensi midgut cells and Plasmodium berghei: the time bomb theory of ookinete invasion of mosquitoes.* EMBO J, 2000. **19**(22): p. 6030-40.

329. Kumar, S., et al., *Inducible peroxidases mediate nitration of anopheles midgut cells undergoing apoptosis in response to Plasmodium invasion.* J Biol Chem, 2004. **279**(51): p. 53475-82.

330. Kumar, S., et al., *A peroxidase/dual oxidase system modulates midgut epithelial immunity in Anopheles gambiae.* Science, 2010. **327**(5973): p. 1644-8.

331. Hillyer, J.F. and T.Y. Estevez-Lao, *Nitric oxide is an essential component of the hemocyte-mediated mosquito immune response against bacteria.* Dev Comp Immunol, 2010. **34**(2): p. 141-9.

332. Peterson, T.M., A.J. Gow, and S. Luckhart, *Nitric oxide metabolites induced in Anopheles stephensi control malaria parasite infection.* Free Radic Biol Med, 2007. **42**(1): p. 132-42.

333. Lim, J., et al., *Induction of nitric oxide synthase in Anopheles stephensi by Plasmodium falciparum: mechanism of signaling and the role of parasite glycosylphosphatidylinositols.* Infect Immun, 2005. **73**(5): p. 2778-89.

334. Akman-Anderson, L., M. Olivier, and S. Luckhart, *Induction of nitric oxide synthase and activation of signaling proteins in Anopheles mosquitoes by the malaria pigment, hemozoin.* Infect Immun, 2007. **75**(8): p. 4012-9.

335. Goncalves, R.L., et al., *Mitochondrial reactive oxygen species modulate mosquito susceptibility to Plasmodium infection.* PLoS One, 2012. **7**(7): p. e41083.

336. Cirimotich, C.M., et al., *Natural microbe-mediated refractoriness to Plasmodium infection in Anopheles gambiae.* Science, 2011. **332**(6031): p. 855-8.

337. Kumar, S., et al., *The role of reactive oxygen species on Plasmodium melanotic encapsulation in Anopheles gambiae.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 14139-44.

338. Molina-Cruz, A., et al., *Reactive oxygen species modulate Anopheles gambiae immunity against bacteria and Plasmodium.* J Biol Chem, 2008. **283**(6): p. 3217-23.

339. Eleftherianos, I. and C. Revenis, *Role and importance of phenoloxidase in insect hemostasis.* J Innate Immun, 2011. **3**(1): p. 28-33.

340. Andersen, S.O., *Insect cuticular sclerotization: a review.* Insect Biochem Mol Biol, 2010. **40**(3): p. 166-78.

341. Christensen, B.M., et al., *Melanization immune responses in mosquito vectors.* Trends Parasitol, 2005. **21**(4): p. 192-9.

342. Nappi, A.J. and B.M. Christensen, *Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity.* Insect Biochem Mol Biol, 2005. **35**(5): p. 443-59.

343. Chen, C.C. and C.S. Chen, *Brugia pahangi: effects of melanization on the uptake of nutrients by microfilariae in vitro.* Exp Parasitol, 1995. **81**(1): p. 72-8.

344. Dubovskiy, I., et al., *Encapsulation and nodulation in insects.* Invertebrate Survival, 2016.

345. Jiang, H. and M.R. Kanost, *The clip-domain family of serine proteinases in arthropods.* Insect Biochem Mol Biol, 2000. **30**(2): p. 95-105.

346. Johnny Nakhleh, L.E.M., Mike A.Osta, *The Melanization Response in Insect Immunity*, in *Advances in Insect Physiology*

. 2017. p. 83-109.

347. Ji, C., et al., *A pattern recognition serine proteinase triggers the prophenoloxidase activation cascade in the tobacco hornworm, Manduca sexta.* J Biol Chem, 2004. **279**(33): p. 34101-6.

348. Roh, K.B., et al., *Proteolytic cascade for the activation of the insect toll pathway induced by the fungal cell wall component.* J Biol Chem, 2009. **284**(29): p. 19474-81.

349. Takahashi, D., B.L. Garcia, and M.R. Kanost, *Initiating protease with modular domains interacts with beta-glucan recognition protein to trigger innate immune response in insects.* Proc Natl Acad Sci U S A, 2015. **112**(45): p. 13856-61.

350. Kanost, M.R. and H. Jiang, *Clip-domain serine proteases as immune factors in insect hemolymph.* Curr Opin Insect Sci, 2015. **11**: p. 47-55.

351. Gulley, M.M., X. Zhang, and K. Michel, *The roles of serpins in mosquito immunology and physiology.* J Insect Physiol, 2013. **59**(2): p. 138-47.

352. Tang, H., et al., *Two proteases defining a melanization cascade in the immune system of Drosophila.* J Biol Chem, 2006. **281**(38): p. 28097-104.

353. Tang, H., et al., *A serpin that regulates immune melanization in the respiratory system of Drosophila.* Dev Cell, 2008. **15**(4): p. 617-26.

354. Nam, H.J., et al., *Genetic evidence of a redox-dependent systemic wound response via Hayan protease-phenoloxidase system in Drosophila.* EMBO J, 2012. **31**(5): p. 1253-65.

355. Dudzic, J.P., et al., *More Than Black or White: Melanization and Toll Share Regulatory Serine Proteases in Drosophila.* Cell Rep, 2019. **27**(4): p. 1050-1061 e3.

356. Yamamoto-Hino, M. and S. Goto, *Spatzle-Processing Enzyme-independent Activation of the Toll Pathway in Drosophila Innate Immunity.* Cell Struct Funct, 2016. **41**(1): p. 55-60.

357. Ma, C. and M.R. Kanost, *A beta1,3-glucan recognition protein from an insect, Manduca sexta, agglutinates microorganisms and activates the phenoloxidase cascade.* J Biol Chem, 2000. **275**(11): p. 7505-14.

358. Jiang, H., et al., *Beta-1,3-glucan recognition protein-2 (betaGRP-2)from Manduca sexta; an acute-phase protein that binds beta-1,3-glucan and lipoteichoic acid to aggregate fungi and bacteria and stimulate prophenoloxidase activation.* Insect Biochem Mol Biol, 2004. **34**(1): p. 89-100.

359. Wang, Y. and H. Jiang, *Interaction of beta-1,3-glucan with its recognition protein activates hemolymph proteinase 14, an initiation enzyme of the prophenoloxidase activation system in Manduca sexta.* J Biol Chem, 2006. **281**(14): p. 9271-8.

360. Wang, Y. and H. Jiang, *Reconstitution of a branch of the Manduca sexta prophenoloxidase activation cascade in vitro: snake-like hemolymph proteinase 21 (HP21) cleaved by HP14 activates prophenoloxidase-activating proteinase-2 precursor.* Insect Biochem Mol Biol, 2007. **37**(10): p. 1015-25.

361. Gorman, M.J., et al., *Manduca sexta hemolymph proteinase 21 activates prophenoloxidase-activating proteinase 3 in an insect innate immune response proteinase cascade.* J Biol Chem, 2007. **282**(16): p. 11742-9.

362. Jiang, H., Y. Wang, and M.R. Kanost, *Pro-phenol oxidase activating proteinase from an insect, Manduca sexta: a bacteria-inducible protein similar to Drosophila easter.* Proc Natl Acad Sci U S A, 1998. **95**(21): p. 12220-5.

363. Jiang, H., et al., *Prophenoloxidase-activating proteinase-2 from hemolymph of Manduca sexta. A bacteria-inducible serine proteinase containing two clip domains.* J Biol Chem, 2003. **278**(6): p. 3552-61.

364. Jiang, H., et al., *Prophenoloxidase-activating proteinase-3 (PAP-3) from Manduca sexta hemolymph: a clip-domain serine proteinase regulated by serpin-1J and serine proteinase homologs.* Insect Biochem Mol Biol, 2003. **33**(10): p. 1049-60.

365. An, C., et al., *Functions of Manduca sexta hemolymph proteinases HP6 and HP8 in two innate immune pathways.* J Biol Chem, 2009. **284**(29): p. 19716-26.

366. Yu, X.Q., et al., *Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, Manduca sexta.* Insect Biochem Mol Biol, 2003. **33**(2): p. 197-208.

367. Gupta, S., Y. Wang, and H. Jiang, *Manduca sexta prophenoloxidase (proPO) activation requires proPO-activating proteinase (PAP) and serine proteinase homologs (SPHs) simultaneously.* Insect Biochem Mol Biol, 2005. **35**(3): p. 241-8.

368. Wang, Y. and H. Jiang, *A positive feedback mechanism in the Manduca sexta prophenoloxidase activation system.* Insect Biochem Mol Biol, 2008. **38**(8): p. 763-9.

369. Wang, Y., Z. Lu, and H. Jiang, *Manduca sexta proprophenoloxidase activating proteinase-3 (PAP3) stimulates melanization by activating proPAP3, proSPHs, and proPOs.* Insect Biochem Mol Biol, 2014. **50**: p. 82-91.

370. Christen, J.M., et al., *Identification of plasma proteinase complexes with serpin-3 in Manduca sexta.* Insect Biochem Mol Biol, 2012. **42**(12): p. 946-55.

371. Wang, Y. and H. Jiang, *Purification and characterization of Manduca sexta serpin-6: a serine proteinase inhibitor that selectively inhibits prophenoloxidase-activating proteinase-3.* Insect Biochem Mol Biol, 2004. **34**(4): p. 387-95.

372. Suwanchaichinda, C., et al., *Manduca sexta serpin-7, a putative regulator of hemolymph prophenoloxidase activation.* Insect Biochem Mol Biol, 2013. **43**(7): p. 555-61.

373. Tong, Y. and M.R. Kanost, *Manduca sexta serpin-4 and serpin-5 inhibit the prophenol oxidase activation pathway: cDNA cloning, protein expression, and characterization.* J Biol Chem, 2005. **280**(15): p. 14923-31.

374. Tong, Y., H. Jiang, and M.R. Kanost, *Identification of plasma proteases inhibited by Manduca sexta serpin-4 and serpin-5 and their association with components of the prophenol oxidase activation pathway.* J Biol Chem, 2005. **280**(15): p. 14932-42.

375. Yang, F., et al., *In search of a function of Manduca sexta hemolymph protease-1 in the innate immune system.* Insect Biochem Mol Biol, 2016. **76**: p. 1-10.

376. Park, J.W., et al., *A synthetic peptidoglycan fragment as a competitive inhibitor of the melanization cascade.* J Biol Chem, 2006. **281**(12): p. 7747-55.

377. Park, J.W., et al., *Clustering of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects.* Proc Natl Acad Sci U S A, 2007. **104**(16): p. 6602-7.

378. Kan, H., et al., *Molecular control of phenoloxidase-induced melanin synthesis in an insect.* J Biol Chem, 2008. **283**(37): p. 25316-23.

379. Lee, K.Y., et al., *A zymogen form of masquerade-like serine proteinase homologue is cleaved during pro-phenoloxidase activation by Ca2+ in coleopteran and Tenebrio molitor larvae.* Eur J Biochem, 2002. **269**(17): p. 4375-83.

380. Zhang, R., et al., *Characterization and properties of a 1,3-beta-D-glucan pattern recognition protein of Tenebrio molitor larvae that is specifically degraded by serine protease during prophenoloxidase activation.* J Biol Chem, 2003. **278**(43): p. 42072-9.

381. Jiang, R., et al., *Three pairs of protease-serpin complexes cooperatively regulate the insect innate immune responses.* J Biol Chem, 2009. **284**(51): p. 35652-8.

382. Zhao, M., et al., *A novel 43-kDa protein as a negative regulatory component of phenoloxidase-induced melanin synthesis.* J Biol Chem, 2005. **280**(26): p. 24744-51.

383. Volz, J., et al., *A genetic module regulates the melanization response of Anopheles to Plasmodium.* Cell Microbiol, 2006. **8**(9): p. 1392-405.

384. Schnitger, A.K., F.C. Kafatos, and M.A. Osta, *The melanization reaction is not required for survival of Anopheles gambiae mosquitoes after bacterial infections.* J Biol Chem, 2007. **282**(30): p. 21884-8.

385. Nakhleh, J., G.K. Christophides, and M.A. Osta, *The serine protease homolog CLIPA14 modulates the intensity of the immune response in the mosquito Anopheles gambiae.* J Biol Chem, 2017. **292**(44): p. 18217-18226.

386. El Moussawi, L., et al., *The mosquito melanization response requires hierarchical activation of non-catalytic clip domain serine protease homologs.* PLoS Pathog, 2019. **15**(11): p. e1008194.

387. An, C., et al., *Characterization of a regulatory unit that controls melanization and affects longevity of mosquitoes.* Cell Mol Life Sci, 2011. **68**(11): p. 1929-39.

388. Zhang, X., et al., *CLIPB8 is part of the prophenoloxidase activation system in Anopheles gambiae mosquitoes.* Insect Biochem Mol Biol, 2016. **71**: p. 106-15.

389. Hernandez-Martinez, S., et al., *Cellular-mediated reactions to foreign organisms inoculated into the hemocoel of Anopheles albimanus (Diptera: Culicidae)*. J Med Entomol, 2002. **39**(1): p. 61-9.

390. Hillyer, J.F., C. Barreau, and K.D. Vernick, *Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel.* Int J Parasitol, 2007. **37**(6): p. 673-81.

391. Hillyer, J.F., S.L. Schmidt, and B.M. Christensen, *Rapid phagocytosis and melanization of bacteria and Plasmodium sporozoites by hemocytes of the mosquito Aedes aegypti.* J Parasitol, 2003. **89**(1): p. 62-9.

392. Hillyer, J.F., S.L. Schmidt, and B.M. Christensen, *Hemocyte-mediated phagocytosis and melanization in the mosquito Armigeres subalbatus following immune challenge by bacteria.* Cell Tissue Res, 2003. **313**(1): p. 117-27.

393. Hillyer, J.F., S.L. Schmidt, and B.M. Christensen, *The antibacterial innate immune response by the mosquito Aedes aegypti is mediated by hemocytes and independent of Gram type and pathogenicity.* Microbes Infect, 2004. **6**(5): p. 448-59.

394. Da Silva, J.B., et al., *Immune defense mechanisms of Culex quinquefasciatus (Diptera: Culicidae) against Candida albicans infection.* J Invertebr Pathol, 2000. **76**(4): p. 257-62.

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

396. Castillo, J.C., A.E. Robertson, and M.R. Strand, *Characterization of hemocytes from the mosquitoes Anopheles gambiae and Aedes aegypti.* Insect Biochem Mol Biol, 2006. **36**(12): p. 891-903.

397. Hillyer, J.F., et al., *Age-associated mortality in immune challenged mosquitoes (Aedes aegypti) correlates with a decrease in haemocyte numbers.* Cell Microbiol, 2005. **7**(1): p. 39-51.

398. Christensen, B.M., et al., *Hemocyte population changes during the immune response of Aedes aegypti to inoculated microfilariae of Dirofilaria immitis.* J Parasitol, 1989. **75**(1): p. 119-23.

399. Bryant, W.B. and K. Michel, *Blood feeding induces hemocyte proliferation and activation in the African malaria mosquito, Anopheles gambiae Giles.* J Exp Biol, 2014. **217**(Pt 8): p. 1238-45.

400. Castillo, J., M.R. Brown, and M.R. Strand, *Blood feeding and insulin-like peptide 3 stimulate proliferation of hemocytes in the mosquito Aedes aegypti.* PLoS Pathog, 2011. **7**(10): p. e1002274.

401. Coggins, S.A., T.Y. Estevez-Lao, and J.F. Hillyer, *Increased survivorship following bacterial infection by the mosquito Aedes aegypti as compared to Anopheles gambiae correlates with increased transcriptional induction of antimicrobial peptides.* Dev Comp Immunol, 2012. **37**(3-4): p. 390-401.

402. Telang, A., et al., *Larval nutritional stress affects vector immune traits in adult yellow fever mosquito Aedes aegypti (Stegomyia aegypti).* Med Vet Entomol, 2012. **26**(3): p. 271-81.

403. Moita, L.F., et al., *Integrins of Anopheles gambiae and a putative role of a new beta integrin, BINT2, in phagocytosis of E. coli.* Insect Biochem Mol Biol, 2006. **36**(4): p. 282-90.

404. Lombardo, F., et al., *Comprehensive genetic dissection of the hemocyte immune response in the malaria mosquito Anopheles gambiae.* PLoS Pathog, 2013. **9**(1): p. e1003145.

405. Sandiford, S.L., et al., *Cytoplasmic actin is an extracellular insect immune factor which is secreted upon immune challenge and mediates phagocytosis and direct killing of bacteria, and is a Plasmodium Antagonist.* PLoS Pathog, 2015. **11**(2): p. e1004631.

406. Blandin, S.A. and E.A. Levashina, *Phagocytosis in mosquito immune responses.* Immunol Rev, 2007. **219**: p. 8-16.

407. Vernick, K.D., et al., *Plasmodium gallinaceum: a refractory mechanism of ookinete killing in the mosquito, Anopheles gambiae.* Exp Parasitol, 1995. **80**(4): p. 583-95.

408. Torii, M., et al., *Penetration of the mosquito (Aedes aegypti) midgut wall by the ookinetes of Plasmodium gallinaceum.* J Protozool, 1992. **39**(4): p. 449-54.

409. Vlachou, D., et al., *Real-time, in vivo analysis of malaria ookinete locomotion and mosquito midgut invasion.* Cell Microbiol, 2004. **6**(7): p. 671-85.

410. Vlachou, D., et al., *Functional genomic analysis of midgut epithelial responses in Anopheles during Plasmodium invasion.* Curr Biol, 2005. **15**(13): p. 1185-95.

411. Mendes, A.M., et al., *Conserved mosquito/parasite interactions affect development of Plasmodium falciparum in Africa.* PLoS Pathog, 2008. **4**(5): p. e1000069.

412. Grimont, F., and Grimont, *The genus Serratia*, in *The Prokaryotes*, S.F. M. Dworkin, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, Editor. 2006, Springer: New York, NY. p. 219–244.

413. Benoit, T.G., et al., *Isolation and pathogenicity of Serratia marcescens from adult house flies infected with Entomophthora muscae.* J Invertebr Pathol, 1990. **55**(1): p. 142-4.

414. Lauzon, C., Bussert, T., Sjogren, R., and Prokopy, R. , *Serratia marcescens as a bacterial pathogen of Rhagoletis pomonella flies (Diptera: Tephritidae)*. Eur. J. Entomol., 2003.

415. Aggarwal, C., Paul, S., Tripathi, V. et al. , *Chitinolytic activity in Serratia marcescens (strain SEN) and potency against different larval instars of Spodoptera litura with effect of sublethal doses on insect development.* Biocontrol, 2015: p. 631–640.

416. Abebe-Akele, F., et al., *Genome sequence and comparative analysis of a putative entomopathogenic Serratia isolated from Caenorhabditis briggsae.* BMC Genomics, 2015. **16**: p. 531.

417. Wang, Y., et al., *Dynamic gut microbiome across life history of the malaria mosquito Anopheles gambiae in Kenya.* PLoS One, 2011. **6**(9): p. e24767.

418. Tchioffo, M.T., et al., *Dynamics of Bacterial Community Composition in the Malaria Mosquito's Epithelia.* Front Microbiol, 2015. **6**: p. 1500.

419. Chandler, J.A., R.M. Liu, and S.N. Bennett, *RNA shotgun metagenomic sequencing of northern California (USA) mosquitoes uncovers viruses, bacteria, and fungi.* Front Microbiol, 2015. **6**: p. 185.

420. Seitz, H.M., et al., *Concomitant infections of Anopheles stephensi with Plasmodium berghei and Serratia marcescens: additive detrimental effects.* Zentralbl Bakteriol Mikrobiol Hyg A, 1987. **266**(1-2): p. 155-66.

421. Rani, A., et al., *Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected Anopheles stephensi-an Asian malarial vector.* BMC Microbiol, 2009. **9**: p. 96.

422. Gonzalez-Ceron, L., et al., *Bacteria in midguts of field-collected Anopheles albimanus block Plasmodium vivax sporogonic development.* J Med Entomol, 2003. **40**(3): p. 371-4.

423. Gusmao, D.S., et al., *First isolation of microorganisms from the gut diverticulum of Aedes aegypti (Diptera: Culicidae): new perspectives for an insect-bacteria association.* Mem Inst Oswaldo Cruz, 2007. **102**(8): p. 919-24.

424. Demaio, J., et al., *The midgut bacterial flora of wild Aedes triseriatus, Culex pipiens, and Psorophora columbiae mosquitoes.* Am J Trop Med Hyg, 1996. **54**(2): p. 219-23.

425. Chen, S., J. Blom, and E.D. Walker, *Genomic, Physiologic, and Symbiotic Characterization of Serratia marcescens Strains Isolated from the Mosquito Anopheles stephensi.* Front Microbiol, 2017. **8**: p. 1483.

426. Labbate, M., et al., *Quorum-sensing regulation of adhesion in Serratia marcescens MG1 is surface dependent.* J Bacteriol, 2007. **189**(7): p. 2702-11.

427. Shanks, R.M., et al., *A Serratia marcescens OxyR homolog mediates surface attachment and biofilm formation.* J Bacteriol, 2007. **189**(20): p. 7262-72.

428. Apte-Deshpande, A., et al., *Serratia odorifera a midgut inhabitant of Aedes aegypti mosquito enhances its susceptibility to dengue-2 virus.* PLoS One, 2012. **7**(7): p. e40401.

429. Bahia, A.C., et al., *Exploring Anopheles gut bacteria for Plasmodium blocking activity.* Environ Microbiol, 2014. **16**(9): p. 2980-94.

430. Bai, L., et al., *A Gut Symbiotic Bacterium Serratia marcescens Renders Mosquito Resistance to Plasmodium Infection Through Activation of Mosquito Immune Responses.* Front Microbiol, 2019. **10**: p. 1580.

431. Bando, H., et al., *Intra-specific diversity of Serratia marcescens in Anopheles mosquito midgut defines Plasmodium transmission capacity.* Sci Rep, 2013. **3**: p. 1641.

432. Castro, A.J., *Antimalarial activity of prodigiosin.* Nature, 1967. **213**(5079): p. 903-4.

433. Azambuja, P., E.S. Garcia, and N.A. Ratcliffe, *Gut microbiota and parasite transmission by insect vectors.* Trends Parasitol, 2005. **21**(12): p. 568-72.

434. Moraes, C.S., et al., *Leishmania (Leishmania) chagasi interactions with Serratia marcescens: ultrastructural studies, lysis and carbohydrate effects.* Exp Parasitol, 2008. **118**(4): p. 561-8.

435. Castro, D.P., et al., *Trypanosoma cruzi: ultrastructural studies of adhesion, lysis and biofilm formation by Serratia marcescens.* Exp Parasitol, 2007. **117**(2): p. 201-7.

436. Mallo, G.V., et al., *Inducible antibacterial defense system in C. elegans.* Curr Biol, 2002. **12**(14): p. 1209-14.

437. Xanthopoulos, C.F.a.K.G., *Insect Pathogenic Properties Of Serratia Marcescens. Passive And Active Resistance To Insect Immunity Studied With Protease-Deficient And Phage-Resistant Mutants.* MICROBIOLOGY 1983. **129**(2).

438. Mochii, M., et al., *Identification of transforming growth factor-beta- regulated genes in caenorhabditis elegans by differential hybridization of arrayed cDNAs.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15020-5.

439. Kurz, C.L., et al., *Virulence factors of the human opportunistic pathogen Serratia marcescens identified by in vivo screening.* EMBO J, 2003. **22**(7): p. 1451-60.

440. Nehme, N.T., et al., *A model of bacterial intestinal infections in Drosophila melanogaster.* PLoS Pathog, 2007. **3**(11): p. e173.

441. Stathopoulos, S., et al., *Genetic dissection of Anopheles gambiae gut epithelial responses to Serratia marcescens.* PLoS Pathog, 2014. **10**(3): p. e1003897.

442. Devenport M, J.-L.M., *The Peritrophic Matrix of Hematophagous Insects*, in *Biology of Disease Vectors*, M. W, Editor. 2005, Elsevier Academic Press: New York. p. 297–310.

443. Huber, M., E. Cabib, and L.H. Miller, *Malaria parasite chitinase and penetration of the mosquito peritrophic membrane.* Proc Natl Acad Sci U S A, 1991. **88**(7): p. 2807-10.

444. Rodgers, F.H., et al., *Microbiota-induced peritrophic matrix regulates midgut homeostasis and prevents systemic infection of malaria vector mosquitoes.* PLoS Pathog, 2017. **13**(5): p. e1006391.

445. Ha, E.M., et al., *A direct role for dual oxidase in Drosophila gut immunity.* Science, 2005. **310**(5749): p. 847-50.

446. Ha, E.M., et al., *Regulation of DUOX by the Galphaq-phospholipase Cbeta-Ca2+ pathway in Drosophila gut immunity.* Dev Cell, 2009. **16**(3): p. 386-97.

447. Ha, E.M., et al., *An antioxidant system required for host protection against gut infection in Drosophila.* Dev Cell, 2005. **8**(1): p. 125-32.

448. Straif, S.C., et al., *Midgut bacteria in Anopheles gambiae and An. funestus (Diptera: Culicidae) from Kenya and Mali.* J Med Entomol, 1998. **35**(3): p. 222-6.

449. Lindh, J.M., O. Terenius, and I. Faye, *16S rRNA gene-based identification of midgut bacteria from field-caught Anopheles gambiae sensu lato and A. funestus mosquitoes reveals new species related to known insect symbionts.* Appl Environ Microbiol, 2005. **71**(11): p. 7217-23.

450. Osei-Poku, J., et al., *Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya.* Mol Ecol, 2012. **21**(20): p. 5138-50.

451. Ngo, C.T., et al., *Bacterial diversity associated with wild caught Anopheles mosquitoes from Dak Nong Province, Vietnam using culture and DNA fingerprint.* PLoS One, 2015. **10**(3): p. e0118634.

452. Tchioffo, M.T., et al., *Modulation of malaria infection in Anopheles gambiae mosquitoes exposed to natural midgut bacteria.* PLoS One, 2013. **8**(12): p. e81663.

453. Kambris, Z., et al., *Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes.* Science, 2009. **326**(5949): p. 134-6.

454. Marois, E., *The multifaceted mosquito anti-Plasmodium response.* Curr Opin Microbiol, 2011. **14**(4): p. 429-35.

455. Inohara, N. and G. Nunez, *ML -- a conserved domain involved in innate immunity and lipid metabolism.* Trends Biochem Sci, 2002. **27**(5): p. 219-21.

456. Zdobnov, E.M., et al., *Comparative genome and proteome analysis of Anopheles gambiae and Drosophila melanogaster.* Science, 2002. **298**(5591): p. 149-59.

457. Kobe, B. and J. Deisenhofer, *The leucine-rich repeat: a versatile binding motif.* Trends Biochem Sci, 1994. **19**(10): p. 415-21.

458. Davies, G. and B. Henrissat, *Structures and mechanisms of glycosyl hydrolases.* Structure, 1995. **3**(9): p. 853-9.

459. Nagai, Y., et al., *Essential role of MD-2 in LPS responsiveness and TLR4 distribution.* Nat Immunol, 2002. **3**(7): p. 667-72.

460. Rodrigues, J., et al., *Hemocyte differentiation mediates innate immune memory in Anopheles gambiae mosquitoes.* Science, 2010. **329**(5997): p. 1353-5.

461. Ramirez, J.L., et al., *A mosquito lipoxin/lipocalin complex mediates innate immune priming in Anopheles gambiae.* Nat Commun, 2015. **6**: p. 7403.

462. Barletta, A.B.F., et al., *Mosquito Midgut Prostaglandin Release Establishes Systemic Immune Priming.* iScience, 2019. **19**: p. 54-62.

463. Castillo, J.C., et al., *Activation of mosquito complement antiplasmodial response requires cellular immunity.* Sci Immunol, 2017. **2**(7).

464. Ayyaz, A., H. Li, and H. Jasper, *Haemocytes control stem cell activity in the Drosophila intestine.* Nat Cell Biol, 2015. **17**(6): p. 736-48.

465. Chakrabarti, S., et al., *Remote Control of Intestinal Stem Cell Activity by Haemocytes in Drosophila.* PLoS Genet, 2016. **12**(5): p. e1006089.

466. Taracena, M.L., et al., *Regulation of midgut cell proliferation impacts Aedes aegypti susceptibility to dengue virus.* PLoS Negl Trop Dis, 2018. **12**(5): p. e0006498.

467. Janeh, M., D. Osman, and Z. Kambris, *Comparative Analysis of Midgut Regeneration Capacity and Resistance to Oral Infection in Three Disease-Vector Mosquitoes.* Sci Rep, 2019. **9**(1): p. 14556.

468. Janeh, M., D. Osman, and Z. Kambris, *Damage-Induced Cell Regeneration in the Midgut of Aedes albopictus Mosquitoes.* Sci Rep, 2017. **7**: p. 44594.

469. Bulet, P. and R. Stocklin, *Insect antimicrobial peptides: structures, properties and gene regulation.* Protein Pept Lett, 2005. **12**(1): p. 3-11.

470. Hanson, M.A., et al., *Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach.* Elife, 2019. **8**.

471. Nakhleh, J., L. El Moussawi, and M.A. Osta, *The melanization response in insect immunity*, in *Insect Immunity*, P. Ligoxygakis, Editor. 2017, Elsevier. p. 2-20.

472. Nappi, A., M. Poirie, and Y. Carton, *The role of melanization and cytotoxic by-products in the cellular immune responses of Drosophila against parasitic wasps.* Advances in Parasitology, 2009. **70**: p. 99-121.

473. King, J.G. and J.F. Hillyer, *Infection-induced interaction between the mosquito circulatory and immune systems.* PLoS Pathog, 2012. **8**(11): p. e1003058.

474. Wood, W. and P. Martin, *Macrophage Functions in Tissue Patterning and Disease: New Insights from the Fly.* Dev Cell, 2017. **40**(3): p. 221-233.

475. Dostalova, A., et al., *Thioester-containing proteins regulate the Toll pathway and play a role in Drosophila defence against microbial pathogens and parasitoid wasps.* BMC Biol, 2017. **15**(1): p. 79.

476. Povelones, M., M.A. Osta, and G.K. Christophides, *The complement system of malaria vector mosquitoes*, in *Progress in Mosquito Research*, A.S. Raikhel, Editor. 2016, Elsevier Ltd. p. 223-242.

477. Vodovar, N., et al., *Drosophila: a polyvalent model to decipher host-pathogen interactions.* Trends in Microbiology, 2004. **12**(5): p. 235-42.

478. Sigle, L.T. and J.F. Hillyer, *Eater and draper are involved in the periostial haemocyte immune response in the mosquito Anopheles gambiae.* Insect Molecular Biology, 2018. **27**(4): p. 429-438.

479. Grimont, P.A. and F. Grimont, *The genus Serratia.* Annual Review of Microbiology, 1978. **32**: p. 221-48.

480. Hejazi, A. and F.R. Falkiner, *Serratia marcescens.* Journal of Medical Microbiology, 1997. **46**(11): p. 903-12.

481. Kurz, C.L., et al., *Virulence factors of the human opportunistic pathogen Serratia marcescens identified by in vivo screening.* EMBO Journal, 2003. **22**(7): p. 1451-60.

482. Pradel, E., et al., *Detection and avoidance of a natural product from the pathogenic bacterium Serratia marcescens by Caenorhabditis elegans.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(7): p. 2295-300.

483. Saab, S.A., et al., *The environment and species affect gut bacteria composition in laboratory co-cultured Anopheles gambiae and Aedes albopictus mosquitoes.* Sci Rep, 2020. **10**(1): p. 3352.

484. Ezraty, B., et al., *Oxidative stress, protein damage and repair in bacteria.* Nat Rev Microbiol, 2017. **15**(7): p. 385-396.

485. Kocks, C., et al., *Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila.* Cell, 2005. **123**(2): p. 335-46.

486. Kurucz, E., et al., *Nimrod, a putative phagocytosis receptor with EGF repeats in Drosophila plasmatocytes.* Current Biology, 2007. **17**(7): p. 649-54.

487. Melcarne, C., et al., *Two Nimrod receptors, NimC1 and Eater, synergistically contribute to bacterial phagocytosis in Drosophila melanogaster.* FEBS J, 2019. **286**(14): p. 2670-2691.

488. Shiratsuchi, A., et al., *Independent recognition of Staphylococcus aureus by two receptors for phagocytosis in Drosophila.* Journal of Biological Chemistry, 2012. **287**(26): p. 21663-72.

489. Piper, R.C. and D.J. Katzmann, *Biogenesis and function of multivesicular bodies.* Annual Review of Cell and Developmental Biology, 2007. **23**: p. 519-47.

490. Golchin, A., S. Hosseinzadeh, and A. Ardeshirylajimi, *The exosomes released from different cell types and their effects in wound healing.* Journal of Cellular Biochemistry, 2018. **119**(7): p. 5043-5052.

491. McCarville, J.L. and J.S. Ayres, *Disease tolerance: concept and mechanisms.* Current Opinion in Immunology, 2018. **50**: p. 88-93.

492. Troha, K., et al., *Comparative transcriptomics reveals CrebA as a novel regulator of infection tolerance in D. melanogaster.* PLoS Pathog, 2018. **14**(2): p. e1006847.

493. Ukleja, M., et al., *Beyond the known functions of the CCR4-NOT complex in gene expression regulatory mechanisms: New structural insights to unravel CCR4-NOT mRNA processing machinery.* Bioessays, 2016. **38**(10): p. 1048-58.

494. Chen, Y., et al., *A DDX6-CNOT1 complex and W-binding pockets in CNOT9 reveal direct links between miRNA target recognition and silencing.* Molecular Cell, 2014. **54**(5): p. 737-50.

495. Yamaguchi, T., et al., *The CCR4-NOT deadenylase complex controls Atg7-dependent cell death and heart function.* Sci Signal, 2018. **11**(516).

496. Morita, M., et al., *Obesity resistance and increased hepatic expression of catabolism-related mRNAs in Cnot3+/- mice.* EMBO Journal, 2011. **30**(22): p. 4678-91.

497. Paul, S., K. Gable, and T.M. Dunn, *A six-membrane-spanning topology for yeast and Arabidopsis Tsc13p, the enoyl reductases of the microsomal fatty acid elongating system.* Journal of Biological Chemistry, 2007. **282**(26): p. 19237-46.

498. Veillard, F., L. Troxler, and J.M. Reichhart, *Drosophila melanogaster clip-domain serine proteases: Structure, function and regulation.* Biochimie, 2016. **122**: p. 255-69.

499. Becker, T., et al., *FOXO-dependent regulation of innate immune homeostasis.* Nature, 2010. **463**(7279): p. 369-73.

500. Fink, C., et al., *Intestinal FoxO signaling is required to survive oral infection in Drosophila.* Mucosal Immunol, 2016. **9**(4): p. 927-36.

501. Mabery, E.M. and D.S. Schneider, *The Drosophila TNF ortholog eiger is required in the fat body for a robust immune response.* J Innate Immun, 2010. **2**(4): p. 371-8.

502. Schneider, D.S., et al., *Drosophila eiger mutants are sensitive to extracellular pathogens.* PLoS Pathog, 2007. **3**(3): p. e41.

503. Guillou, A., et al., *The Drosophila CD36 Homologue croquemort Is Required to Maintain Immune and Gut Homeostasis during Development and Aging.* PLoS Pathog, 2016. **12**(10): p. e1005961.

504. Ramet, M., et al., *Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria.* Immunity, 2001. **15**(6): p. 1027-38.

505. Stuart, L.M., et al., *Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain.* Journal of Cell Biology, 2005. **170**(3): p. 477-85.

506. Martins, N.E., et al., *Host adaptation is contingent upon the infection route taken by pathogens.* PLoS Pathog, 2013. **9**(9): p. e1003601.

507. Mondotte, J.A., et al., *Immune priming and clearance of orally acquired RNA viruses in Drosophila.* Nat Microbiol, 2018. **3**(12): p. 1394-1403.

508. Carissimo, G., et al., *Highly focused transcriptional response of Anopheles coluzzii to O'nyong nyong arbovirus during the primary midgut infection.* BMC Genomics, 2018. **19**(1): p. 526.

509. Hughes, L.J., et al., *Magnitude and diversity of immune response to vaccinia virus is dependent on route of administration.* Virology, 2020. **544**: p. 55-63.

510. Demars, A., et al., *Route of Infection Strongly Impacts the Host-Pathogen Relationship.* Front Immunol, 2019. **10**: p. 1589.

511. Wong, A.C., et al., *Gut Microbiota Modifies Olfactory-Guided Microbial Preferences and Foraging Decisions in Drosophila.* Current Biology, 2017. **27**(15): p. 2397-2404 e4.

512. Cole, J.N. and V. Nizet, *Bacterial Evasion of Host Antimicrobial Peptide Defenses* Microbiology Spectrum, 2016. **4**(1): p. 1-40.

513. Choe, K.M., H. Lee, and K.V. Anderson, *Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(4): p. 1122-6.

514. Iatsenko, I., et al., *PGRP-SD, an Extracellular Pattern-Recognition Receptor, Enhances Peptidoglycan-Mediated Activation of the Drosophila Imd Pathway.* Immunity, 2016. **45**(5): p. 1013-1023.

515. Nakhleh, J., G.K. Christophides, and M.A. Osta, *The serine protease homolog CLIPA14 modulates the intensity of the immune response in the mosquito Anopheles gambiae.* Journal of Biological Chemistry, 2017. **292**(44): p. 18217-18226.

516. Sousa, G.L., et al., *The CLIP-domain serine protease CLIPC9 regulates melanization downstream of SPCLIP1, CLIPA8, and CLIPA28 in the malaria vector Anopheles gambiae.* BioRxiv, 2020.

517. Severo, M.S., et al., *Unbiased classification of mosquito blood cells by single-cell genomics and high-content imaging.* Proceedings of the National Academy of Sciences of the United States of America, 2018. **115**(32): p. E7568-E7577.

518. Muller, H.M., et al., *A hemocyte-like cell line established from the malaria vector Anopheles gambiae expresses six prophenoloxidase genes.* J Biol Chem, 1999. **274**(17): p. 11727-35.

519. Buchon, N., et al., *Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation.* Cell Host Microbe, 2009. **5**(2): p. 200-11.

520. Chakrabarti, S., et al., *Infection-induced host translational blockage inhibits immune responses and epithelial renewal in the Drosophila gut.* Cell Host Microbe, 2012. **12**(1): p. 60-70.

521. Becher, P.G., et al., *Yeast, not fruit volatiles mediate Drosophila melanogaster attraction, oviposition and development* Funct Ecol, 2012. **26**(4): p. 822-828.

522. Zhu, J., K.C. Park, and T.C. Baker, *Identification of odors from overripe mango that attract vinegar flies, Drosophila melanogaster.* J Chem Ecol, 2003. **29**(4): p. 899-909.

523. Scopelliti, A., et al., *A Neuronal Relay Mediates a Nutrient Responsive Gut/Fat Body Axis Regulating Energy Homeostasis in Adult Drosophila.* Cell Metab, 2019. **29**(2): p. 269-284 e10.

524. Song, W., et al., *Midgut-Derived Activin Regulates Glucagon-like Action in the Fat Body and Glycemic Control.* Cell Metab, 2017. **25**(2): p. 386-399.

525. Lee, K.Z., et al., *Enterocyte Purge and Rapid Recovery Is a Resilience Reaction of the Gut Epithelium to Pore-Forming Toxin Attack.* Cell Host Microbe, 2016. **20**(6): p. 716-730.

526. Nsango, S.E., et al., *Genetic clonality of Plasmodium falciparum affects the outcome of infection in Anopheles gambiae.* International Journal for Parasitology, 2012. **42**(6): p. 589-95.

527. Nsango, S.E., et al., *AP-1/Fos-TGase2 axis mediates wounding-induced Plasmodium falciparum killing in Anopheles gambiae.* Journal of Biological Chemistry, 2013. **288**(22): p. 16145-54.

528. Krautz, R., B. Arefin, and U. Theopold, *Damage signals in the insect immune response.* Front Plant Sci, 2014. **5**: p. 342.

529. Bidla, G., et al., *Hemolymph coagulation and phenoloxidase in Drosophila larvae.* Developmental and Comparative Immunology, 2005. **29**(8): p. 669-79.

530. Franz, A., W. Wood, and P. Martin, *Fat Body Cells Are Motile and Actively Migrate to Wounds to Drive Repair and Prevent Infection.* Dev Cell, 2018. **44**(4): p. 460-470 e3.

531. Galko, M.J. and M.A. Krasnow, *Cellular and genetic analysis of wound healing in Drosophila larvae.* PLoS Biol, 2004. **2**(8): p. E239.

532. Goto, A., T. Kadowaki, and Y. Kitagawa, *Drosophila hemolectin gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects.* Developmental Biology, 2003. **264**(2): p. 582-91.

533. Karlsson, C., et al., *Proteomic analysis of the Drosophila larval hemolymph clot.* J Biol Chem, 2004. **279**(50): p. 52033-41.

534. Lesch, C., et al., *A role for Hemolectin in coagulation and immunity in Drosophila melanogaster.* Developmental and Comparative Immunology, 2007. **31**(12): p. 1255-63.

535. Losick, V.P., D.T. Fox, and A.C. Spradling, *Polyploidization and cell fusion contribute to wound healing in the adult Drosophila epithelium.* Current Biology, 2013. **23**(22): p. 2224-2232.

536. Scherfer, C., et al., *Isolation and characterization of hemolymph clotting factors in Drosophila melanogaster by a pullout method.* Curr Biol, 2004. **14**(7): p. 625-9.

537. Scherfer, C., et al., *The Toll immune-regulated Drosophila protein Fondue is involved in hemolymph clotting and puparium formation.* Dev Biol, 2006. **295**(1): p. 156-63.

538. Jung, J., H. Zeng, and T. Horng, *Metabolism as a guiding force for immunity.* Nat Cell Biol, 2019. **21**(1): p. 85-93.

539. Wang, A., H.H. Luan, and R. Medzhitov, *An evolutionary perspective on immunometabolism.* Science, 2019. **363**(6423).

540. O'Neill, L.A. and E.J. Pearce, *Immunometabolism governs dendritic cell and macrophage function.* Journal of Experimental Medicine, 2016. **213**(1): p. 15-23.

541. Pietri, J.E., et al., *Two insulin-like peptides differentially regulate malaria parasite infection in the mosquito through effects on intermediary metabolism.* Biochemical Journal, 2016. **473**(20): p. 3487-3503.

542. Pakpour, N., et al., *Ingested human insulin inhibits the mosquito NF-kappaB-dependent immune response to Plasmodium falciparum.* Infection and Immunity, 2012. **80**(6): p. 2141-9.

543. Musselman, L.P., et al., *A Complex Relationship between Immunity and Metabolism in Drosophila Diet-Induced Insulin Resistance.* Molecular and Cellular Biology, 2018. **38**(2).

544. Cheon, H.M., et al., *Regulation of lipid metabolism genes, lipid carrier protein lipophorin, and its receptor during immune challenge in the mosquito Aedes aegypti.* J Biol Chem, 2006. **281**(13): p. 8426-35.

545. Oliveira, J.H., et al., *Energy metabolism affects susceptibility of Anopheles gambiae mosquitoes to Plasmodium infection.* Insect Biochemistry and Molecular Biology, 2011. **41**(6): p. 349-55.

546. Lampe, L., et al., *Metabolic balancing by miR-276 shapes the mosquito reproductive cycle and Plasmodium falciparum development.* Nat Commun, 2019. **10**(1): p. 5634.

547. Eming, S.A., T.A. Wynn, and P. Martin, *Inflammation and metabolism in tissue repair and regeneration.* Science, 2017. **356**(6342): p. 1026-1030.

548. Vodovar, N., et al., *Drosophila host defense after oral infection by an entomopathogenic Pseudomonas species.* Proc Natl Acad Sci U S A, 2005. **102**(32): p. 11414-9.

549. Liehl, P., et al., *Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model.* PLoS Pathog, 2006. **2**(6): p. e56.

550. Ryu, J.H., et al., *An essential complementary role of NF-kappaB pathway to microbicidal oxidants in Drosophila gut immunity.* EMBO J, 2006. **25**(15): p. 3693-701.

551. Kounatidis, I. and P. Ligoxygakis, *Drosophila as a model system to unravel the layers of innate immunity to infection.* Open Biol, 2012. **2**(5): p. 120075.

552. Basset, A., et al., *The phytopathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3376-81.

553. Buchon, N., et al., *Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila.* Genes Dev, 2009. **23**(19): p. 2333-44.

554. Limmer, S., et al., *Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a Drosophila melanogaster oral infection model.* Proc Natl Acad Sci U S A, 2011. **108**(42): p. 17378-83.

555. Joyner, J., et al., *Use of quantitative real-time PCR for direct detection of serratia marcescens in marine and other aquatic environments.* Applied and Environmental Microbiology, 2014. **80**(5): p. 1679-83.

556. Patro, R., et al., *Salmon provides fast and bias-aware quantification of transcript expression.* Nat Methods, 2017. **14**(4): p. 417-419.

557. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biol, 2014. **15**(12): p. 550.

558. Young, M.D., et al., *Gene ontology analysis for RNA-seq: accounting for selection bias.* Genome Biol, 2010. **11**(2): p. R14.

559. Benjamini, Y. and Y. Hochberg, *Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing.* J R Stat Soc Series B, 1995. **57**: p. 289-300.

560. Habtewold, T., et al., *Transmission blocking immunity in the malaria non-vector mosquito Anopheles quadriannulatus species A.* PLoS Pathog, 2008. **4**(5): p. e1000070.

# APPENDIX