Examination of Toll pathway interactions during innate immune responses in the mosquito *Aedes aegypti*

by

Cassidy Nathan Garry Erdelyan

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University of Manitoba

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Abstract

Like other insects, mosquitoes rely on innate immunity pathways to provide protection from a diversity of pathogens. As vectors of some of our most serious diseases, mosquitoes must protect themselves from viral (e.g. dengue, West Nile virus) or protozoan (e.g. malaria) pathogens they transmit, and they must also defend themselves from opportunistic bacterial and fungal pathogens that they can acquire following accidental breaks or wounds in their cuticle. Among the insect defense mechanisms is the Toll-mediated pathway, which chiefly protects against Gram-positive bacterial and fungal pathogens, but can also induce antiviral defenses.

In this study, responses of the Toll pathway in the mosquito *Aedes aegypti* to two different pathogens were examined, with the aim to identify the role of some of the components of the extracellular signaling cascade that activates Toll. In the first part of this study, the interaction between one of the mosquito's extracellular signaling cascade proteins, modular serine protease (modSP), and a protein of West Nile virus, non-structural protein 1 (NS1) was examined. NS1 in vertebrates can interact with complement proteins to down-regulate the immune responses, and in a previous yeast two-hybrid screen, NS1 was observed to interact with modSP. To assess this interaction and a possible immune modulatory function in the mosquito, the proteins were over-expressed in insect cell cultures and antibody-mediated pull-down methods were used to detect interactions. Conflicting results were obtained using two different antibodies, and consequently, the interaction remains unconfirmed.

In the other part of this study, the mosquito's response to a Gram-positive bacterium *Staphylococcus epidermidis* was examined using a transcriptomic approach. Over 40 known or predicted immune response genes were up-regulated following the infection. RNA interference

(RNAi)-mediated knockdown of a few genes confirmed their involvement in the immune responses, but their precise roles in the signaling pathway will require further examination.

These findings illustrate that RNA sequencing, coupled with RNAi validation techniques, could provide valuable insights into the adaptations and dynamic nature of the immune system in mosquitoes and could also provide new targets for another generation of mosquito control technologies.

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Chapter 1: Introduction

Mosquitoes are our most serious disease vectors, transmitting a range of arboviruses, protozoan parasites, nematodes, and bacteria, all which pose major risks to humans and livestock, infecting millions of people every year[1]. Malaria is the most serious mosquito-borne pathogen, infecting an estimated 219 million people, and resulting in 435,000 deaths in 2017[2]. Flaviviruses are the most common mosquito-borne arboviruses, and include the well-known Zika (ZIKV), West Nile (WNV) and dengue (DENV) viruses, the latter of which infects 390 million people each year[3]. For many of these diseases, we lack effective vaccines, and hence, most of our efforts to manage the diseases have focused on controlling mosquito populations. As a result of extensive use of chemical pesticides over the past six decades, many of the vector species have developed insecticide resistance[4] and many current mosquito research efforts are focused on finding new approaches to control the most serious disease-vectoring species.

Not all mosquito species, however, pose a threat to human or livestock health. What defines a species as a serious vector is based on its specific host preferences, its distribution patterns, and on its capacity, or vector competence, to propagate and transmit different pathogens[5]. Vector competence not only differs across species, but can also vary among populations of the same species, making this aspect of the mosquitoes' biology even more challenging to solve [6,7]. The ability of a mosquito species or population to transmit pathogens is dependent on many factors related to the host-pathogen interactions. In general, species that are considered effective disease vectors are presumed to suffer limited or minimal impacts from the pathogen. Otherwise, the insect would either succumb to the infection before it can effectively

transmit the pathogen to its new host, or it may fail to reproduce, thereby limiting the pathogen's spread in subsequent generations[8,9].

Clearly, mosquitoes must defend themselves against the pathogens they carry. While there are innumerable studies that have explored the impacts of the pathogens and their host vertebrate defense responses, there is still much that we need to learn about how mosquitoes deal with the pathogens they can carry. Malaria, for example, is a disease caused by an apicomplexan protozoan that is transmitted to humans only by mosquitoes of the genus *Anopheles*. While we know that the parasite can proliferate within the mosquito, moving from midgut to salivary gland cells over the course of the infection within the insect, we still have little understanding of whether the parasite seriously affects the mosquito[10,11].

Understanding impacts of the pathogens on insects requires a better understanding of the insects' natural defenses against the invasive microorganisms. While insects lack adaptive (antibody-mediated) immunity, they do possess innate immunity systems that can protect them from serious damage from various pathogens. To defend against physical breaks of their tough cuticle, insects will rely on coagulation, melanization, hemocyte degranulation, and scar formation to provide a level of physical protection against the pathogens. But once pathogens have broken through these defenses, insects will rely upon their innate immune system, which is comprised of cellular and humoral components to neutralize the invading pathogens. Much of what we know about insect innate immunity responses is based on studies of a limited number of insect species, including the silk moth *Bombyx mori,* the tobacco hornworm *Manduca sexta*, the mealworm *Tenebrio molitor*, and the fruit fly *Drosophila melanogaster*. *Drosophila* is by far the most thoroughly studied insect, due largely to its well-annotated genome, thousands of mutant

strains, and the diversity of genetic manipulation methods, including genetic transformation tools that have been developed first in this species[12].

While this review focuses primarily on the innate immunity processes within mosquitoes, it will also examine what is known about similar processes in *Drosophila* and a limited number of other insects, to highlight differences in immunity mechanisms where they exist, or to highlight what more we need to know about mosquito immunity if we are to find alternative approaches to limiting their ability to spread disease.

Physical barriers:

Cuticle and epithelial cells

The cuticle is the hard exoskeleton of the insect and is made primarily of layers of the polysaccharide chitin cross-linked with various proteins such as sclerotin and anthropodin. The outer layer of the cuticle is covered with a thin layer of cross-linked lipoproteins (mostly cuticulin) and crystalline waxes that forms a water-proof barrier. Together, this multilayered structure forms a protective barrier against physical damage and prevents pathogens easy access to the mosquito's underlying tissues. The components of the cuticle are secreted by specialized epithelial cells attached to a basement membrane. As the exoskeleton must bend at joints and in between body segments, there are thinner regions where this outer barrier may be more prone to damage or pathogen invasion.

Midgut, hemocoel, salivary glands

The other primary route of entry for pathogens in insects is the alimentary canal. The epithelial cells that line the alimentary canal comprise the so-called midgut barrier. The cells themselves may prevent pathogens from entering the insect's hemocoel, but a subset of epithelial cells also secrete an additional barrier known as the peritrophic membrane (PM).

The PM is a semi-permeable physical barrier that surrounds the food bolus within the digestive tract. By enclosing the newly ingested food, the PM can prevent physical abrasions of the intestinal epithelial cells from large food particles, and with pore sizes generally between 2-10 nm, it allows the free movement of digestive enzymes into the bolus and digested macromolecules to escape and be absorbed in larval mosquitoes[13]. As adults, only a blood-fed female produces a PM[13].The PM is composed of chitin fibrils and glycoproteins such as peritrophins that protect the midgut epithelial cells and help prevent infection by large pathogens when a mosquito takes a bloodmeal[14,15]. The PM also protects the mosquito from heme released from digested blood, which is a toxic oxidizer to mosquitoes, damaging lipids, protein and DNA; some PM proteins have been shown to sequester heme in *Ae. aegypti,* resulting in the excretion of the majority of this toxic compound[16–18].

A loss-of-function mutant of Drosocrystallin (*dcy*) in *Drosophila* shows an increased permeability of the peritrophic matrix and increased susceptibility to an infection delivered orally and not via a septic injury[19]. If a pathogen successfully passes through the peritrophic matrix, the midgut cells are the first point of infection in mosquitoes[20]. The *Plasmodium* parasite, for example, has developed mechanisms to weaken the PM. *Plasmodium* ookinetes secrete chitinase to help them escape the PM[21], while simultaneously increasing the rate of bloodmeal digestion in

a mosquito[22,23]. Some *Plasmodium* strains can also secrete pro-chitinase that is activated by mosquito trypsin when the mosquito feeds on a bloodmeal, thereby enabling the pathogen to pass through the PM more readily[23]. While an intact PM may retard a protozoan parasite such as *Plasmodium*, the PM does little to prevent arboviral infections; virions have been found to infect midgut cells within the first hour, before a PM is fully formed[15].

Once past the PM, the pathogens must next pass across the mosquito midgut barrier, the epithelial cell layer within the gut. For many mosquito-borne pathogens, the midgut cells serve as the first point of infection (i.e. replication) within the mosquito. Some viruses appear well adapted to defend against the midgut cells' defenses. DENV's NS1 protein, for example, can help the virus overcome the mosquito midgut barrier by decreasing the ROS (reactive oxygen species) and other innate immunity responses while also increasing commensal bacteria in the mosquito gut[24]. The importance of the midgut barrier in DENV competence was recently demonstrated in a strain of *Ae. aegypti* with reduced DENV competency. These partially-resistant mosquitoes were observed to have a higher midgut cell proliferation rate that was stimulated by ROS[25]. Activating intestinal stem cell division seems to play an important role in the competency of mosquitoes to DENV. Susceptible strains that are induced to have more regeneration and cell turnover occurring in their gut are refractory to infection while strains with reduced turnover show higher rates of infection.

Commensal gut microorganisms:

Infection of the mosquito gut epithelial cells may not be wholly dependent on the pathogen itself, but may also be dependent on other microorganisms within the mosquito gut. For example, *Talaromyces* fungi within the gut were found to increase mosquito susceptibility to

DENV infection. Secretions from the fungus were observed to down-regulate release of blood-digesting enzymes in the midgut while also impairing trypsin via an unknown factor[26]. Evidence that gut serine proteases can limit viral infections was subsequently confirmed by RNAi-mediated knockdown of trypsin 5G1, which resulted in increased mosquito susceptibility to DENV[27,28].

Gut bacteria may also play a role in mediating viral and pathogen infections of the midgut cells. Wu *et al.* observed that antibiotic-treated *Ae. aegypti* were less susceptible to DENV. They discovered that *Serratia marcescens* digests mucins coating the midgut, thereby increasing DENV infection[29]. *S. odorifera* can also enhance DENV infection, apparently by blocking prohibitin, which the authors postulated is a non-receptor molecule[30], as knockdown of prohibitin leads to higher ROS production and can affect various cellular functions[31]. The importance of this protein in the viral infection process is supported by additional experiments, where knockdown and antibody-blocking of prohibitin resulted in reduced DENV infection rates[32]. In *An. gambiae*, microbiota also play a role in the refractoriness of a Plasmodium infection due to ROS production affecting development before midgut invasion[33]. A recently discovered bacterium *Chromobacterium Csp_P* is entomopathogenic but, inhibits other gut microbiota, reduces *Ae. aegypti*'s susceptibility to both Plasmodium and DENV, and has anti-pathogen activity from an unknown stable secondary metabolite[34].

Hemocoel Defenses:

Insects have an open circulatory system where the hemolymph directly bathes organs and tissues. Within the hemolymph is a variety of hemocytes that represent the cellular components of the innate immunity defenses of insects. Pathogens that are ingested and cross the midgut or

enter through a gap/injury of the cuticle are disseminated by the hemolymph and are exposed to hemocytes. While in the hemocoel, hemocytes can phagocytize, lyse or melanize the pathogen[35]. The majority of circulating hemocytes are granulocytes, which can phagocytose pathogens and small particles, can lyse pathogens, and can also assist oenocytoids, which produce phenoloxidase and other enzymes in the melanization pathway[35]. Prohemocytes may result from an uneven division of granulocytes and are also phagocytic. In some insects, additional hemocytes, such as crystal cells and lamellocytes in *Drosophila*, and spherule and plasmatocytes in *Culex* have been identified, and can contribute to the repertoire of cellular responses to bacterial infections[36,37].

Salivary glands:

Only female mosquitoes require a blood meal, as they require this protein source to help nourish their developing eggs within their ovaries. To acquire the blood meal, they bite various vertebrate hosts, depending on each species' host preferences, and the salivary glands of the mosquito are essential for the blood-feeding process. Saliva is injected into the host, delivering various inhibitors to prevent blood coagulation[38]. Saliva thereby facilitates feeding, by regulating host blood vessel vasoconstriction and inflammation processes. Pathogens transmitted by mosquitoes must therefore migrate to and penetrate the salivary gland to facilitate their escape from the mosquito[39]. The salivary gland is yet one more barrier within the insect that can impact arbovirus infectivity[40].

Entry of pathogens into salivary glands is a receptor-mediated process for the malaria parasite[41] and similarly, there is evidence that there are also receptors on salivary glands for DENV infection[42]. While it is understood that DENV must replicate and accumulate in salivary

glands to deliver an effective infectious dose[43], little is known about the molecular interactions of the virus and mosquito host, and whether multiple molecules are mediating the mosquito's vector competence of different pathogens[44]. The ability of the mosquito to restrict movements of the infectious agents has not been adequately explored, particularly in terms of the mosquito's own defenses to protect itself from the pathogens.

Physiological barriers:

The innate immunity system of insects detects a broad group of pathogen-associated molecular patterns (PAMPs), which are molecular non-self-patterns or molecules that are produced by the pathogen, including lipopolysaccharide, peptidoglycan and β-(1,3)-glucans[45,46]. These PAMPs are recognized by both membrane-bound and soluble pattern recognition receptors (PRRs)[47]. Some PRRs recognize bacterial peptidoglycans and lipopolysaccharides or β-(1,3)-glucans from fungi.

Among the various humoral responses that can respond to these PAMPs is the pro-phenol oxidase (PPO) proteolytic cascade, which can protect the insect from pathogens in two ways: 1) by producing a variety of quinone substances and reactive oxygen intermediates that kill invading pathogens; and 2) by producing melanin to encapsulate the pathogens and prevent their proliferation[48, 49]. Circulating hemocytes are generally considered to produce PPO[35,48] but, depending on the insect, PPO can be found in different hemocytes such as prohemocytes, granulocytes, plasmatocytes, oenocytoids and crystal cells[50]. In *Culex quinquefasciatus*, PPO-positive cells can vary depending on the developmental stage and if the female has blood-fed[51].

In *Drosophila*, the melanization cascade affects resistance to and tolerance of pathogens[52], but flies lacking a functional PPO cascade can nevertheless survive microbial infections[53]. Melanization proteases such as MP1 and MP2 are induced by and can encapsulate infectious fungi. MP1 was also observed to facilitate encapsulation of bacteria in *Drosophila*, but it was not required for the survival of the insects, as other immune functions can play more substantial roles in protection against bacteria[54].

In addition to the PPO system, numerous pattern recognition proteins are used by insects to activate immune pathways such as Imd (Immune deficiency) and Toll. The Imd pathway responds primarily to Gram-negative and some Gram-positive bacterial infections, while Toll responds to Gram-positive bacteria and fungi. These pathways are activated by two major PRRs, β-glucan recognition proteins (βGRPs) and Gram-negative binding proteins (GNBPs), which are both part of the same receptor family and were first discovered in *Bombyx mori*[55]. They were named after the discovery that they bind β-(1,3)-glucans from fungi[46,56,57] and peptidoglycan (PG) from Gram-negative bacteria[58], respectively.

Three GNBPs have been described in *Drosophila*, belonging to the GNBP/β-glucan recognition proteins (βGRP) family[55]. While they do not bind Gram-negative bacteria, they are similar in sequence to *Bombyx*'s GNBPs[59]. *Drosophila*'s GNBP3 is most similar to the lepidopteran β-(1,3)-glucan recognition proteins, and once bound to β-(1,3)-glucan, it activates the Toll pathway for fungal defense[60]. In *Drosophila*, GNBP1 targets Gram-positive bacteria PGs[61,62]. The role that GNBP2 plays is currently unknown[63]. While Bacillus bacteria are Gram-positive, they resemble Gram-negative bacteria in that their PG has a diaminopimelic acid (DAP)

at the third residue instead of lysine, and consequently, Bacillus bacteria will interact with GNBPs to activate the Imd pathway in *Drosophila*[64].

Immune Pathway Signaling – including JAK-Stat, Imd, Toll

Following the initial binding of the PRRs to their respective PAMPs, one or more different signaling pathways can be activated, leading to the production of antimicrobial peptides (AMPs) or induction of other protective cellular responses. The three main pathways associated with AMP production are the Imd, the Jak-STAT, and the Toll pathways, each of which play roles in attacking invasive bacteria and/or fungal pathogens.

Jak-STAT

The Janus kinase/signal transducer and activator of transcription (Jak-STAT) pathway has been less studied than the Toll and Imd pathways in insects, and the mechanisms of induction of this innate immunity pathway are still not fully understood[65]. The first evidence of Jak-STAT immune responses in insects were observed in *An. gambiae* after bacterial challenges, where AgSTAT proteins were observed to translocate to the nucleus following infection[66]. In *Drosophila*, some of the Jak-STAT pathway components have been identified, and include the extracellular Unpaired proteins that interact, in as yet to be defined mechanisms, with PRRs, which then bind to Domeless and Janus tyrosine kinase receptors (Hopscotch or Hop), which then activate the intracellular STAT proteins to enter the nucleus to initiate gene expression, producing AMPs[65]. In *Drosophila*, Jak-STAT has been observed to contribute to host defense during infection with the Gram-negative bacterium *Serratia marcescens*. In the infected flies, the PPO response initiates an oxidative burst, which in turn induces the Jak-STAT pathway

coincident with gut stem cell proliferation[67,68]. Septic injury in *Drosophila* has also been observed to induce hemocyte-activation of Jak-STAT to produce various AMPs[69].

In *An. gambiae*, fungal infections of *Beauveria bassiana* can activate Jak-STAT and contribute to anti-fungal defense[65]. While Jak-STAT can be activated by both fungi and bacteria, there is also evidence that it can act as an antiviral defense[70], although the precise mechanisms of antiviral mechanisms have not yet been fully elucidated[20]. In the mosquito *Ae. aegypti*, for example, RNAi-mediated depletion of two Jak-STAT pathway components, Dome and Hop, leads to increased DENV infection, while depletion of a negative regulator of the pathway, PIAS, leads to increased resistance to the virus. Interestingly, mosquitoes overexpressing Dome or Hop were more resistant to DENV infection, yet their susceptibility to two related flaviviruses, ZIKV or CHIKV, was not affected[71].

Imd

Another distinct innate immunity pathway, the so-called immune deficiency (Imd) pathway, is induced by Gram-negative bacteria[72]. These pathogens' peptidoglycans (PGs) are recognized by a family of PG-recognizing proteins (PGRPs), which can act both as amidases to degrade the PGNs and as inducers of signal transduction pathways and proteolytic cascades. Insect PGRPs are classified as either short (PGRP-S) or long (PGRP-L)[73]; short PGRPs are secreted while long PGRPs can be intracellular, extracellular, or membrane-bound. The signaling cascades initiated by different PGRPs will vary, but in general, they are mediated by three NF-κB transcription factor paralogues, Dorsal (DL), Dorsal-related immunity factor (DIF), and Relish (Rel), the first two inducing the Toll pathway while the third activates the Imd pathway.

Relish is a transcription factor that can initiate transcription of a diverse array of AMPs, and in *Ae. aegypti*, Imd/Relish knockout leads to increased susceptibility to Gram-negative infections[74].

PGRP-LC is one example of a transmembrane signal transducer that ultimately activates the Imd transcription factor Relish[75]. In *Drosophila*, flies deficient in PGRP-LC are extremely susceptible to infection by Gram-negative bacteria and not Gram-positive or fungal infections[76,77]. PGRP-LC may also be involved in phagocytosis of Gram-negative and not Gram-positive bacteria in both *Drosophila*[77] and *An. gambiae*[78].

The Imd pathway not only deals with bacterial infections, but it can assist with eukaryotic parasites. In *An. gambiae*, PGRP-LC also plays a role in removing a large portion of malaria parasites after a bloodmeal. The microbiota rapidly divide after the nutrient rich bloodmeal, leading to an immune response which also eliminates most malaria parasites and appears to mediated by the PGRP-LC3 isoform[79]. In *An. gambiae*, RNAi-mediated silencing of Caspar, a negative regulator of Relish, overexpresses REL2 and leads to Plasmodium resistance[80].

PGRPs are not however, exclusively activating Toll or Imd pathways in insects. PGRP-LE, for example, is a soluble PGRP that on one hand activates Imd/Rel, but it also activates ProPO in *Drosophila* larvae[81,82] and is also responsible for autophagy of Listeria infections, independent of Toll and Imd pathway signaling[83].

Toll

Toll and Imd are the two major insect immune pathways and in many infections, the two pathways can interact[84]. In past years, the immune system was thought to consist of individual and independent components, but increasing evidence shows that there is considerable cross-talk

between the different immune pathways[85–88]. Toll was first identified as an essential component of dorsal ventral (DV) patterning in embryonic development in *Drosophila melanogaster*[89] and was later identified as having a role in the immune system and hemocytes, as overexpression of Toll led to increased expression of the bacteriocidal peptide cecropin[90]. In *Drosophila*, Toll doesn't bind the pathogen like mammalian Toll-like receptors, but instead is activated by a cascade of serine proteases much like the coagulation or complement activation pathways[91]. Toll and Spz were then identified to be important in the antifungal response and production of drosomycin[92] and also implicated in the fungal response of *Aedes*[93]. Mutants deficient in Toll and Imd do not produce AMPs and are therefore susceptible to bacterial and fungal infections[92].

While the Toll pathway has been moderately well characterized in a select few species of insects, the pathway is still not fully elucidated, and can vary greatly among insects. Virtually no proteins between the receptors and Toll in the Toll pathway have been experimentally validated in mosquitoes[94]; the PGRPs are the best characterized members in the Toll pathway in various insects, with little known of the rest of the pathway's components[95–97](Figure 1).

In *Drosophila*, two PGRPs are involved in Toll signaling, PGRP-SA and -SD, both of which are soluble, circulating in the hemolypmh[98,99], and are involved in the detection of Gram-positive bacteria, recognizing the lysine-type PG[64]. PGRP-SA (also known as semmelweis, or seml) is the first PRR upstream of Toll and is produced in the fat body and hemocytes[100]. GNBP1 and PGRP-SA form a complex with PG[61,62]. GNBP1 hydrolyzes the PG for PGRP-SA to bind and the PG enhances GNBP1/PGRP-SA interaction[101,100]. PGRP-SD enhances both GNBP1 binding PG and GNBP1/PGRP-SA binding[102]. While fungal pathogens can activate Toll, they do not activate PGRP-SA[98].

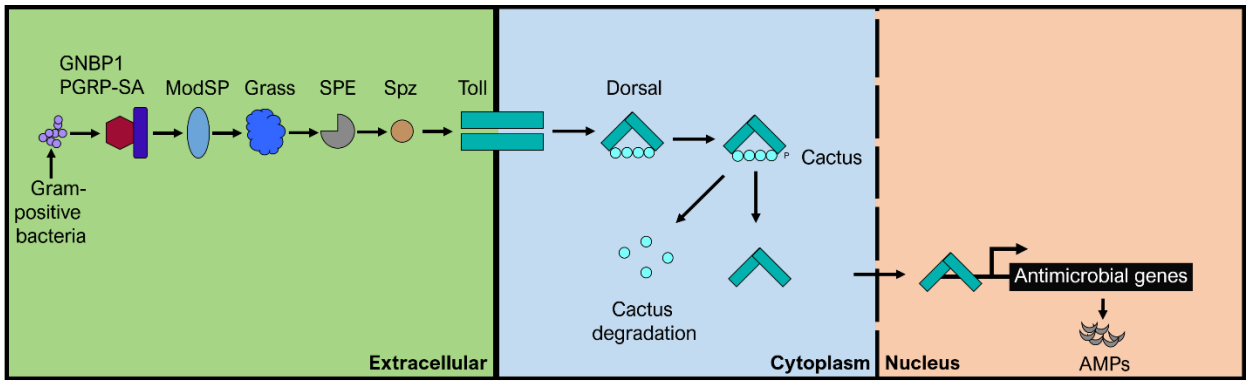


Figure 1. Schematic of a simplified version of the Toll pathway from the detection of Gram-positive bacteria to the transduction intracellularly and antimicrobial peptide transcription in the nucleus of an immune cell (Adapted from Kounatidis and Ligoxygakis, 2012[103]).

A cascade of serine proteases is involved in connecting GNBPs to the activation of Toll. In *Drosophila*, among the first of these is modular serine protease (modSP), which when activated by GNBP1 or GNBP3, then activates Gram-positive-specific serine protease (Grass), activating Spätzle-processing enzyme (SPE)[91]. SPE finally activates Spätzle (Spz)[104], the protein that binds to Toll. In other insects, homologues for only some of these serine proteases have been identified, and it is unclear whether the missing counterparts are unnecessary or simply have not been identified. This cascade of serine proteases is considered our best understanding of a complex network of interactions involved in regulating Toll signaling. Other detection systems also seem to feed into the serine protease cascade. For example, pathogen-derived proteins, broadly defined as virulence factors, can initiate the so-called danger signal cascade, which can independently activate SPE. This pathway is mediated by a circulating serine protease, Persephone (Psh), which is itself activated by some bacterial or fungal proteases[105]. It has been suggested that Psh can effectively bait exogenous proteases[106] leading to SPE-activation of Toll[105,106]. Interestingly, if the pathogens are heat-killed, Psh does not interact with SPE,

resulting in a GNBP-induced pathway that is not accelerated by Psh. Flies with a loss of Psh died from fungal infection but were not affected by Gram-positive bacterial infections[107].

The extracellular cascade of serine proteases that leads to activation of Toll is also closely regulated by a superfamily of serine proteinase inhibitors known as serpins. Serpins possess a functionally conserved loop near the carboxyl-terminus that binds to the protease's active site. When the protease attempts to cut the loop, the serpin undergoes a conformational change, and in a state of partial hydrolysis, the serpin-protease complex is trapped in a covalent complex that effectively inhibits the protease. The role of serpins in modulating immune responses was clearly demonstrated in the beetle *Tenebrio molitor*, where it was found that one serpin functioned as inducible negative feedback inhibitor by inhibiting each of the three serine proteases in the Toll pathway[108,109]. Serpins have since been described in many insects, including mosquitoes, where between 18 to 31 serpins have been identified in different species[110]. Serpins are important negative regulators of not just the Toll signaling pathway, but they also downregulate prophenoloxidase and melanization immune responses. This diversity of serpins has not been fully explored, and their precise functions have yet to be elucidated, but they are generally viewed as an important component of the immune response pathways, ensuring that the responses do no exceed the extent of infection.

Antimicrobial Peptides (AMPs)

Ultimately, once Spz binds to Toll, Toll integrates the extracellular signal it receives and transmits the signal intracellularly, resulting in the activation of Toll-induced signaling complex (TICS), which is comprised of MyD88, Tube, and Pelle. The activated TICS phosphorylates Cactus, which in turn results in the ubiquitination and ultimate destruction of Cactus. Cactus is a

negative regulator of dorsal and DIF (dorsal-related immunity factor), and hence, the destruction of Cactus enables dorsal and DIF to translocate to the nucleus, where they act as transcription factors binding to κB-like regions of DNA leading to the up-regulated expression of various AMP genes[111–113]. The upstream sequences of these genes contain transcription activator binding sites for Dorsal, DIF, and Rel proteins in *Drosophila*[111,112,114] and *Aedes*[115,116].

AMPs have been most thoroughly described in *Drosophila* and include: drosomycin (fungal), cecropins (Gram-negative), drosocin (Gram-negative), metchnikowin (fungi and Gram-positive), defensin (Gram-positive), diptericin (Gram-negative) and attacin (Gram-negative)[117,118]. *Ae. aegypti* has homologues of many of the AMPs in *Drosophila*, including defensins, cecropins, diptericin, and attacin, but they also have an AMP only found in mosquitoes, gambicin which is regulated by Toll, Imd, and JAK-STAT pathways in Aag2 cells[119].

A single AMP may, on occasion, be sufficient to eliminate an infection, but typically, numerous AMPs are produced to provide full protection against a microorganism. Many AMPs targeting Gram-negative bacteria are usually generated simultaneously, where they appear to function additively rather than synergistically to clear out the infection[120]. Most AMPs affect the microorganism's membrane, causing it to depolarize, leak electrolytes or nutrients, leading to death[121]. Cecropins, for example, can cause leaky membranes and depolarization, ultimately leading to lysis of bacterial membranes[121], while defensins form pore-like structures in the bacterial cell membrane[121], also leading to cytoplasmic leakage and cell death. Attacins, in contrast, can block outer-membrane protein synthesis in Gram-negative bacteria[121], resulting in overall malfunctioning of the nutrient uptake and ion balances.

Research Objectives:

There has been considerable research on uncovering the molecular mechanisms of innate immune responses in mosquitoes, but nevertheless, we still do not have a clear idea of whether these responses can fully suppress an infection or can simply delay proliferation of the pathogen until sometime after the reproductive phase of the insect. Given that mosquitoes have been and continue to be vectors of some of our most serious diseases, the insects obviously tolerate the pathogens they transmit, but it is not clear whether the insects' immune system is activated against all of these infectious agents, and whether the pathogens have the ability to either evade or suppress the insects' defenses.

Over the course of my research, I focused on two main research themes that were aimed at exploring the mosquito's responses to two different pathogens. The objectives of each theme are briefly outlined below. Further elaboration of each theme's objectives will be provided in each of the respective chapters that follow.

Theme 1. Evaluating the interaction of West Nile virus NS1 with Aedes aegypti's modSP.

West Nile virus (WNV) is a relatively recently introduced mosquito-borne virus to North America, having first arrived in 1999, but quickly spreading across most of the continent within a few years. WNV infections in humans can range from completely symptomless cases to more extreme cases of encephalitis and death. One intriguing protein of WNV is NS1, which can down-regulate the host's immune system to facilitate a more pronounced infection. In a previous study from our lab, a yeast two-hybrid screen was performed to identify which proteins within the mosquito *Aedes aegypti* interacted with WNV NS1. Intriguingly, one protein that appeared to

interact with NS1 was modSP, which is one of the extracellular proteases that can activate the Toll immune pathway in insects.

In this current study, I set out to examine whether this NS1-modSP interaction could be confirmed by producing variants of both NS1 and modSP, with the goal of elucidating whether NS1 also played an immunomodulatory role in the mosquito, thereby facilitating viral transmission.

Theme 2. Evaluating the transcriptomic responses of Aedes aegypti to an infection of a Gram-positive bacterium, Staphylococcus epidermidis.

The Toll immunity pathway in insects is typically triggered in response to Gram-positive bacterial and fungal infections[113]. The Toll pathway has been moderately well defined in the model insect *Drosophila melanogaster*, and putative homologues of some of the core components of the Toll signaling pathway have been identified in other insects such as the beetle *Tenebrio molitor*[122] and the moth *Bombyx mori*[123,124]. In these insects, and presumably in mosquitoes, bacterial or fungal PAMPs are first detected by PGRP and GNBP extracellular receptors, and through a cascade of serine proteases, including the aforementioned modSP, Spaetzle is activated to bind to the Toll receptor on immune-responsive cells in the insects. Toll then initiates an intracellular signaling cascade to induce the production of AMPs.

In mosquitoes, many of the intracellular components of the Toll signaling pathway have been identified, but the extracellular signaling cascade has not been fully elucidated. In this study, I set out to conduct a transcriptomic analysis of mosquitoes infected with an inducer of the Toll pathway, *Staphylococcus epidermidis*. The aim of the study was to identify a broader suite

of genes responding to the infection than previously examined, and possibly identify other members of the serine protease cascade working with modSP in regulating immune responses. I also used RNA interference techniques to knockdown the expression of some of the newly identified genes in the mosquitoes, to confirm which genes were critical in providing protection to the insect during the infection.

Chapter 2: Examination of potential interactions of the West Nile virus NS1 protein with the mosquito immune response protein, modSP

Introduction

Mosquitoes are vectors of many flaviviruses, the most serious being dengue virus (DENV), which is endemic in over 100 tropical and subtropical countries, causing approximately 500,000 cases of serious hemorrhagic fever and more than 20,000 deaths annually[125]. Other mosquito-borne flaviviruses, such as yellow fever virus (YFV), Chikungunya virus, Zika virus and West Nile virus (WNV) are not as deadly as dengue, but nevertheless, each has caused considerable concern and burdens on our health care systems. Of these flaviviruses, WNV is currently considered the most life-threatening to North American populations[126].

West Nile virus was first introduced to North America in 1999, with the first cases reported in New York City, but over the next several years, WNV infections were detected throughout most parts of North America. In WNV infections, most patients are symptomless, with the young and old being more susceptible to disease[127]. The majority of cases are patients displaying symptoms typical of WN fever, which can include fever, headache, myalgia and gastrointestinal symptoms. More serious forms of the disease can manifest as WN meningitis and even WN encephalitis, which can result in death[128]. The first reported cases of WNV infections in Canada occurred in 2002, and since its arrival, there have been a total of 96 deaths in Canada attributed to this virus. Annual infection rates are variable, but tend to rise during rainy summer years that favour rapid increases in mosquito populations (https://ipac-canada.org/west-nile-virus-resources.php).

In North America, *Culex* species of mosquitoes are the main vectors of WNV, but over 70 different species, including those of other genera, can be infected[129,130]. Female mosquitoes become infected with WNV following ingestion of a blood meal from an infected vertebrate host, typically birds, which serve as the feral reservoir for this virus. Entry of flaviviruses into cells is mediated by one of its structural proteins, the E glycoprotein[131,132]. The WNV E glycoprotein has been observed to interact with a variety of different host cell glycoproteins or lipoproteins, including the lectin DC-SIGN[131], $\alpha_V\beta_3$ integrin[132], and others (reviewed in Rey, 2003[133]), suggesting that there is no single common receptor protein for all flaviviruses.

WNV is similar in structure to the other flaviviruses, having three structural (C, M, and E) and seven nonstructural proteins (NS1, NS2A/B, NS3, NS4A/B, NS5), translated as a single polyprotein from a (+)ssRNA genome and cleaved by host and viral proteases. The structural proteins form the coat of the infectious virion while the nonstructural proteins are primarily involved in replication of the virus within the host cells[134,135].

Among the nonstructural proteins, NS1 has been associated with the additional ability to modulate the vertebrate's host immune system. Infected vertebrate cells can either secrete or present on the cell surface the NS1 protein, which is thought to attenuate complement activation by binding and recruitment of the complement regulatory factor H[136]. DENV, WNV and YFV NS1 can also recruit and complex with C4 and the protease C1s, and thereby promote degradation of C4 to C4b to diminish complement activation[137]. NS1 can thereby ultimately protect the virus and infected cells from immune recognition and neutralization in vertebrates.

While there have been many studies examining the interactions of WNV impacts and the immunomodulatory role of NS1 in vertebrates, nothing is known about possible impacts or

interactions of NS1 in the vector mosquitoes. However, in a yeast two-hybrid screen of WNV proteins interactions with *Ae. aegypti* mosquito proteins conducted in our laboratory, a direct interaction was observed between the WNV NS1 protein and a regulatory protein of the mosquito's innate immunity response, modSP, was observed (Scott Read, MSc thesis).

ModSP is a serine protease that plays a role in insects' innate immunity by activating the Toll pathway, typically in response to infections with Gram-positive bacteria and fungi[91]. The Toll pathway, however, has also been implicated in antiviral responses in the model insect *Drosophila melanogaster*, and it may serve a similar role in mosquitoes[87]. The interaction of NS1 and modSP in the mosquitoes has not been validated beyond the yeast two-hybrid analyses, and hence, the impact that NS1 may have on the mosquito's immune system has yet to be clarified.

In this chapter, I describe efforts to confirm the interaction of the WNV NS1 and *Ae. aegypti*'s modSP proteins, with the aim to evaluate whether the virus is capable of modulating the vector's immune system, as it can in the vertebrate host.

Methods

Insect rearing

Aedes aegypti mosquitoes were reared at 28°C with a 16:8 h light:dark cycle with 60% relative humidity. Eggs were hatched in deionized water and larvae were fed ground liver powder and rabbit food pellets. Adults were fed ad libitum 10% sucrose water and females were blood-fed once weekly on warmed (42°C) rat blood sealed within two layers of stretched Nescofilm (Karlan Research Products).

Expression of NS1 proteins

WNV NY99 RNA was kindly provided by M. Drebot (National Microbiology Laboratory) and cDNA was prepared using an RNA Extraction kit (Qiagen). The full length NS1 protein sequence was PCR amplified using the following primers WNV E KpnI F (GTATAGGTACCATGGAYAGGTCCATAGCTCTCACG) and WNV NS1 XbaI R with the addition of a stop codon (GTATATCTAGATTAAGCATTCACTTGTGACTGCAC). The PCR products were resolved by agarose electrophoresis, gel extracted using the QIAquick Gel Extraction kit (Qiagen), and then ligated into pCDNA 3.1(+) (Thermo Fisher Scientific) using T4 Ligase (Thermo Fisher Scientific). As an untagged vector, the KpnI site was used as a Kozak sequence with the addition of a start codon and 24 amino acids were included from the E glycoprotein, which is sufficient as the secretory signal for NS1[138–140].

A longer variant of NS1, NS1', which is occasionally produced by neuroinvasive flaviviruses that is 52 amino acids longer, was generated by PCR. Primers modeled off a paper producing JEV NS1'[141] failed while another group's approach targeting the slippery heptanucleotide (YCCTTTT) in Kunjin virus, an attenuated form of WNV[142], succeeded. The primers WNV NS1' F (ATGATTGACCC**A**TTCTCAGTTGGGCCT) and WNV NS2a XbaI R (GTATATCTAGATTAGTGTAAGTAATGCCCCCAAAC) to create the C-terminal fragment and, WNV E KpnI F and WNV NS1' R (AGGCCCAACTGAGAA**T**GGGTCAATCAT) to create the N-terminal fragment. Both fragments were resolved, gel extracted, and then combined and used as template with WNV E KpnI F and WNV NS2a XbaI R to generate full length WNV NS1'. The WNV NS1' primers being reverse complements, introduce an adenosine nucleotide (**bolded**) to disrupt the predicted pseudoknot structure and alter the heptanucleotide sequence

(Figure 2), resulting in a frameshifted sequence mimicking the -1 ribosomal frameshift[143], which when expressed, would produce NS1' protein of 404 amino acids in length, compared to the NS1 protein, which is 352 amino acids long (Figure 3). The PCR products were resolved by agarose electrophoresis, gel extracted using the QIAquick Gel Extraction Kit (Qiagen), and then ligated into pcDNA3.1(+).

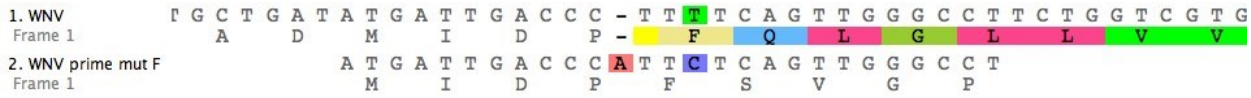


Figure 2. Alignment of WNV NY99 sequence with WNV NS1' F to disrupt the heptanucleotide sequence occurring in NS2a.

Figure 3. Alignment of WNV NS1 (352 aa) and NS1' (404 aa).

The expression plasmids were then transfected into HEK293T cells using TurboFect Transfection Reagent (Thermo Fisher Scientific). Cells were then grown at 37°C and 5% CO_2 for 2-4 days. Cells were recovered from the flasks by scraping and subsequently pelleted with centrifugation. The supernatant was brought to a concentration of 25 mM imidazole and NS1 was affinity purified using Ni-NTA Agarose (Qiagen) according to the manufacturer's instructions.

WNV NS1 BbsI F (GTATAGAAGACATAGGTGACACTGGRTGTGCCATAGAC) was used with the appropriate R primer to amplify WNV NS1 and NS1' fragments, digested with BbsI (New England Biolabs) and cloned into pE-SUMO3-kan (LifeSensors Inc) using BsaI (New England Biolabs). The primers and plasmid contain class IIS restriction enzyme sites, enabling asymmetric cleaving of a unique sequence downsteam of the recognition site[144]. The

plasmids were transformed into BL21-Gold(DE3) (Agilent Technologies), grown to OD_{600} of 0.4 and induced by 1 mM IPTG, grown at 28°C or 16°C overnight with shaking. The pE-SUMO3 plasmids were also transformed into Shuffle T7 *E. coli* cells (New England Biolabs). Induced by 0.4 mM IPTG, grown at 28°C or 16°C overnight with shaking. NS1 is insoluble when expressed in *E. coli*, forming inclusion bodies.[145,146]. SUMO helps recalcitrant proteins remain active and soluble[147] while Shuffle expresses chaperones and creates an oxidizing environment more similar to the endoplasmic reticulum[148]. Induced *E. coli* expressing NS1 were sonicated in TNG (50 mM Tris, pH 7.4, 0.1 M NaCl, and 10% glycerol) buffer and pelleted or, disrupted using a French press in 50 mM Tris, pH 8, 1 M NaCl buffer, with the pellet being resuspended in TNG. Samples were diluted in Laemmli loading buffer and 5% 2-mercaptoethanol. NS1 was affinity purified using Ni-NTA Agarose (Qiagen) to affinity purify the His-tagged proteins according to the manufacturer's instructions.

The NS1 gene fragments were ligated into pM-secSUMOstar (LifeSensors Inc) using BsmBI (New England Biolabs), pM-secSUMOstar has a pcDNA3 backbone and includes a mouse IgG κ secretory signal and a cleavable SUMOstar 6xHis tag to facilitate purification. Plasmids were transfected as pcDNA 3.1(+) above.

Additionally, the NS1 gene fragments were ligated into pI-SecSUMOstar (LifeSensors Inc), an insect cell vector for production of secreted recombinant proteins with a cleavable 6xHis-SUMOstar tag in a baculovirus expression system. A similar method has been used before in Sf9 cells[149]. The pI-SecSUMOstar plasmids were transformed into DH10Bac *E. coli* cells (Thermo Fisher Scientific). These cells have a baculovirus shuttle vector (bMON14272) and a helper plasmid (pMON7142), which together support site-specific recombination between the

pFastBac-based pI-SecSUMOstar plasmid and bMON14272 to generate bacmids. The resulting bacmids, expressing each of the two NS1 variants, were transfected into Sf9 (*Spodoptera frugiperda*) cells (Thermo Fisher Scientific), which support baculovirus replication and production of the recombinant proteins using Cellfectin II Reagent (Thermo Fisher Scientific) and grown at ambient room temperature or 27°C with no additional CO_2.

A myc tag (EQKLISEEDL) was added to pI-SecSUMOstar and pM-SecSUMOstar by using Phusion High-Fidelity DNA polymerase as it is a non-strand displacing polymerase[150], with 5'-phosphorylated primers myc pisecsumostar phos F (TCCGAGGAGGACCTGGGGTCCCTGCAGGACTCAG) and myc pisecsumostar phos R (GATCAGCTTCTGCTCGTGATGATGATGGTGATGACCC) for pI-SecSUMOstar and, myc pmsecsumostar phos F (TCCGAGGAGGACCTGGGGTCCCTGCAGGACTCA) and myc pmsecsumostar phos R (GATCAGCTTCTGCTCGTGATGATGATGGTGATGACCG) for pM-SecSUMOstar. FastDigest DpnI (Thermo Fisher Scientific) was used to cleaved methylated parental DNA and the PCR products were resolved by agarose electrophoresis, gel extracted using the QIAquick Gel Extraction kit (Qiagen), and then ligated to eliminate transformation of the parental strains.

Expression of Aedes aegypti modSP protein

To ensure that mosquitoes were expressing modSP, adults were fed for 4 days on fungus-contaminated cotton swabs dipped in 10% sucrose.RNA was extracted from pools of 5 adult mosquitoes using an RNA extraction kit (Qiagen) and cDNA was prepared using SuperScript First-Strand cDNA Synthesis kit (Invitrogen). The modSP coding sequence was PCR amplified using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) using primers Ae

modsp BsmBI F (GTATACGTCTCAAGGTATGCAAAGATATTCATTCTTTCGAG) and Ae modsp BsmBI R (GTATACGTCTCTCTAGTCATATCACAGGGAATCGACTTTC). A ΔmodSP clone, carrying a deletion of the complement control protein (CCP) modules was generated by PCR using overlapping primers Ae modSP d215 F (ACCTGTTGGATCCGAACGTTACTGTT) and Ae modSP d215 R (AACAGTAACGTTCGGATCCAACAGGT) containing a deletion and gel purifying fragments before subjecting them to PCR using the original forward and reverse primers (Figure 4).

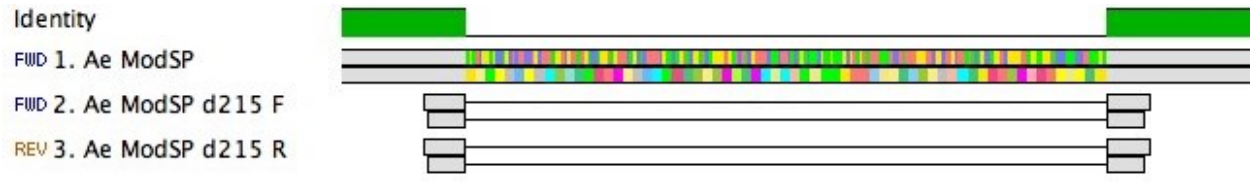


Figure 4. Schematic of primers spanning CCP region of modSP to introduce a deletion corresponding to amino acids 215-279, creating ΔmodSP.

To evaluate whether the N- or the C-terminus of modSP interacts with NS1 and the effect they have on the insect immune system, N- and C-terminal modSP proteins were generated using the following primers: Ae modsp BsmBI F and Ae modsp N BsmBI R (GTATACGTCTCTCTAGTCAGTATGCTTCAGCATCTGGCGT) to create the N-terminus along with Ae modsp C BsmBI F (GTATACGTCTCAAGGTATTATTGGAGGTCGAAATGCC) and Ae modsp BsmBI R to create the C-terminus. Each of the modSP variants (full length modSP, ΔmodSP, and the N-and C-terminal regions) were ligated into the plasmid pI-SecSUMOstar, transformed into DH10Bac *E. coli* cells, and the resultant bacmids produced were used to transfect Sf9 insect cells, as described above.

To generate N- and C-terminal uniquely tagged modSP, HA and V5 tags were added to the N- and C-terminus respectively using primers pI Ae V5 N term modsp F (CTCCTCGGTCTCGATTCTACGATGCAAAGATATTCATTCTTTCGAGAC)and pI V5 N term modsp R (AGGGTTAGGGATAGGCTTACCACCTCCAATCTGTTCTCTGTGAGC) and, pI HA C term modsp F (TCCAGATTACGCTTGACTAGAGCGGCCGCGGTA) and pI Ae HA C term modsp R (ACATCGTATGGGTATATCACAGGGAATCGACTTTCGG) as described above to create myc-tagged pI-SecSUMOstar. ModSP was similarly purified using magnetic beads as described above.

Western blotting analysis of protein expression

Media and transfected cells were harvested 2-5 days post-transfection. Cells were lysed in NP-40 buffer and purified using Pierce Protein A/G Magnetic Beads (Thermo Fisher Scientific). An attempt was made using a His antibody (HIS.H8, Thermo Fisher Scientific) cross-linked to the Protein A/G beads using DSS (Thermo Fisher Scientific). NS1 was eluted using 0.1 M glycine, pH 2.0 and neutralized with phosphate buffer. Subsequent attempts used the 9E10 anti-myc antibody (kindly provided by Peter Pelka, University of Manitoba) or the 4G4 anti-NS1 mouse monoclonal antibody (kindly provided by Roy Hall, University of Queensland) according to the manufacturer's instructions but after washing, the beads were suspended in Laemmli loading buffer and 5% 2-mercaptoethanol.

Protein samples were diluted in Laemmli loading buffer and 5% 2-mercaptoethanol, then boiled for 10 minutes. Proteins were resolved using SDS-PAGE (12% acrylamide) and transferred to PVDF membranes (Millipore) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad).

As the modSP proteins carried myc tags, 9E10 was used to detect this protein on the blots. To detect untagged NS1 and NS1' proteins, 4G4 was used.

An alternative antibody to modSP was also prepared, as cleavage of the SUMO tag would render the protein undetectable. A polyclonal antibody to detect modSP was prepared by Genscript, using a fragment of the *Culex quinquefasciatus* modSP protein sequence (IIGGKNASIAEVPWHC), rather than the corresponding *Ae. aegypti* modSP sequence (IIGG**R**NA**T**I**T**EVPWHC, bolding indicating the differences), as the *Culex* modSP was predicted by Genscript to be more immunogenic.

Western blot membranes were blocked with 5% skim milk in PBST, and incubated with the appropriate primary antibodies (9E10 or anti-*Culex* modSP for modSP detection; 9E10 or 4G4 for tagged or untagged NS1 detection) in blocking solution plus primary antibody overnight at 4˚C. Membranes were washed in PBST, and the goat-anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) (Thermo Fisher Scientific) was applied for 1 hour at room temperature. Following three washes in in PBST, secondary antibodies were detected using Lumina Forte Western HRP substrate (Millipore), and chemiluminescence was detected using a C-DiGit Blot Scanner (LI-COR) or X-ray film and a developer.

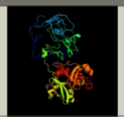
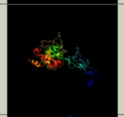
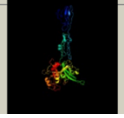
Results

Initial attempts to PCR amplify full length *Ae. aegypti* modSP using EconoTaq polymerase (Lucigen) or Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) failed, while positive control PCR amplifications of *PIAS* from this cDNA were successful. It was suspected that the primers required to attain a full length CDS were poor, there were few

modSP transcripts from the mosquitoes used for this initial attempt or, intact full-length transcripts in the cDNA were in low abundance, and hence, the mosquitoes were fed 10% sucrose solutions on cotton swabs contaminated with fungus growth, in an effort to induce an immune response to generate more transcripts along with a range of amplification temperatures and Mg^{2+} concentrations. The modSP gene fragment was successfully isolated using this method.

An examination of the predicted structure of the mosquito modSP protein suggests that it contains complement control protein (CCP) modules/SUSHI repeats. A BLASTP[151] search of these sequences showed high identity (22-25%) to modules found in C4b and Complement Factor H (fH), while the entire modSP protein was most similar to Complement Factor I (Figure 5) with 95% of the protein modelled at >90% confidence using Phyre2[152]. Mannan-binding lectin serine proteases are also involved in the complement pathway[153], though no interaction has been identified with MASP and NS1, MASP binds E[154] and, NS1 binds to mannose-binding lectin to prevent neutralization by the complement pathway[155].

A

#	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
1	c2xrcD_	Alignment		100.0	25	**PDB header:**immune system **Chain:** D: **PDB Molecule:**human complement factor i; **PDBTitle:** human complement factor i
2	c1zjkA_	Alignment		100.0	27	**PDB header:**hydrolase **Chain:** A: **PDB Molecule:**mannan-binding lectin serine protease 2; **PDBTitle:** crystal structure of the zymogen catalytic region of human masp-2
3	c4kkdB_	Alignment		100.0	24	**PDB header:**hydrolase **Chain:** B: **PDB Molecule:**mannan-binding lectin serine protease 1; **PDBTitle:** the x-ray crystal structure of mannose-binding lectin-associated2 serine proteinase-3 reveals the structural basis for enzyme3 inactivity associated with the 3mc syndrome

B

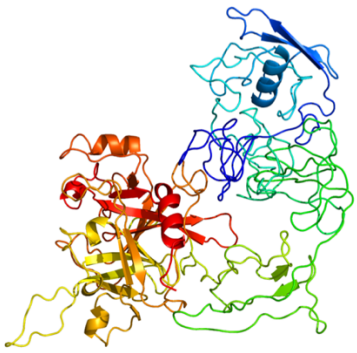


Figure 5. Phyre2 modeling of *Ae. aegypti* modSP protein based on structural data. A) Template B) Structural model.

Interestingly, Complement Factor H from bovine serum and purified human fH have been previously shown to interact with WNV NS1 generated from human, insect and hamster cells[136]. NS1 also interacts directly with C4b[156]. These observations suggest that NS1 might be interacting with modSP in the mosquito in an analogous manner to the way that NS1 interacts with complement factors in vertebrates. Given that NS1 can act in an immunomodulatory manner in vertebrates, through interactions with complement proteins, most likely through CCP regions[157], there is a possibility that NS1 is also modulating the innate immunity pathway in the mosquito, through its interaction with modSP.

To express the variants of NS1 and modSP proteins, a HEK293 mammalian cell expression system was first used with plasmids that would produce untagged NS1. Multiple attempts were made to isolate sufficient proteins to be detected on western blots, but yields were typically low to completely undetectable and using a finite supply of antibody.

An alternative method of producing NS1 was therefore developed, a baculovirus expression system, using Sf9 (*Spodoptera frugiperda*; fall armyworm moth) cells, as these insect cells were considered to be a more appropriate system to express the insect modSP proteins, and baculovirus expression systems have the potential to generate larger yields of secreted protein than plasmid expression systems. Using this expression system, both modSP and NS1 proteins were produced from Sf9 cells (Figure 6).

To examine whether the mosquito modSP interacts directly with WNV NS1, I attempted to co-purify NS1 interacting with modSP. An attempt to cross-link NS1 antibody to the A/G beads was made, but a western blot failed to detect NS1 after elution, electrophoresis, and

blotting. Even though the proteins were meant to be secreted into the medium (Figure 6A), the proteins were recovered mostly in the cell lysate extracts (Figure 6B).

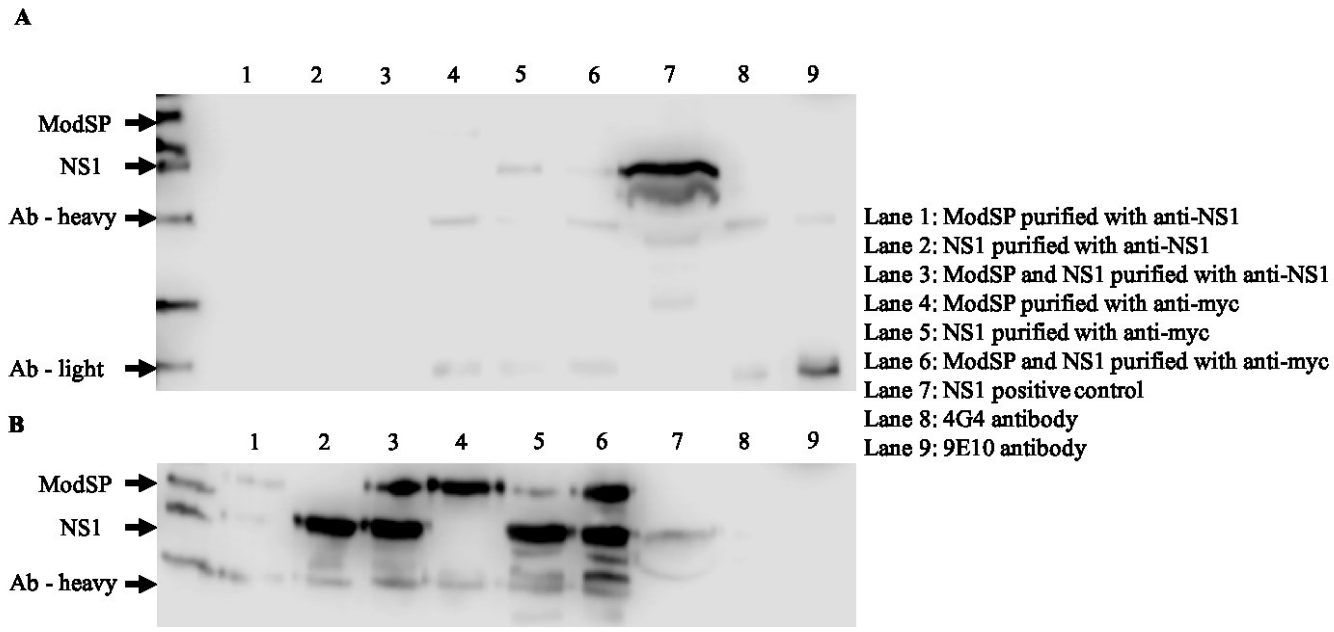


Figure 6. Western blot of A) protein purified from supernatant and B) protein purified from cell lysate of baculovirus infected Sf9 cells. Proteins were purified using magnetic Protein A/G beads with either 4G4 (anti-NS1) or 9E10 (anti-myc) antibodies and detected using 9E10.

Yields were still low, as the chemiluminescence signal from the secondary antibodies was often undetected directly from the probed western blots unless 9E10 antibodies were used. Only by using X-ray film, with lengthy exposures to amplify the signal, could the proteins be consistently detected when using 4G4 or anti-*Culex* modSP (Figure 7), suggesting that this expression system would require considerable optimization if sufficient proteins were to be isolated for quantitative analyses of interaction between the various NS1 and modSP variant proteins.

Figure 7. Western blot of a modSP protein sample loaded in duplicate and incubated with either A) 9E10 anti-myc or B) anti-*Culex* modSP primary antibody.

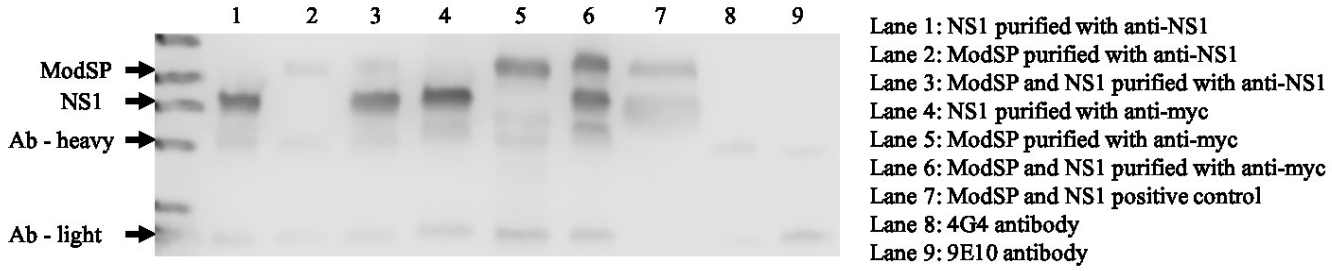


Figure 8. Western blot of protein purified from cell lysate of baculovirus infected Sf9 cells. Proteins were purified using magnetic Protein A/G beads with either 4G4 (anti-NS1) or 9E10 (anti-myc) antibodies and detected using 9E10.

Based on the blot in Figure 6B, the two bands in lane 3 suggest that modSP can bind to NS1, as the modSP and NS1 proteins were co-purified using the anti-NS1 antibody. However, as there was a small amount of protein visible in lane 1, there is the possibility that some spill-over of samples may have occurred in other lanes of this gel. Hence, this entire experiment was repeated, and in Figure 8, lane 3 shows that only the faintest trace of modSP is visible in the samples purified with the anti-NS1 antibody. This same amount of modSP is present in lane 2, which did not have NS1 added, which suggests that modSP was binding weakly to the anti-NS1 beads, even without NS1 protein present. These two results, with their contradictory results, made it impossible to conclude whether modSP binds to NS1. More experiments were planned to test whether one or both halves of modSP (N-and C-terminal halves) bind NS1, but as the other components of this research were demanding more attention (see Chapter 3), the experiments remain to be completed.

Discussion

In this study, the potential of WNV's NS1 protein to interact with modSP from *Ae. aegypti* was examined using a pull-down assay that would assess whether the two proteins could bind each other in an *in vitro* format. Using a baculovirus expression system to produce each protein, enough of each protein was produced to conduct the pull-down experiments. Disappointingly, due to conflicting results, the interaction of modSP and NS1 proteins could not be confirmed. Further efforts to optimize the expression constructs, interaction assays, and detection methods were initiated, but it was recognized that these efforts might require many months, and other components of the research were beginning to yield intriguing results. Hence, the additional experiments planned to explore the interaction of NS1 and modSP were terminated.

A wide range of platforms is available for production of recombinant proteins, and each system has its own advantages and limitations. Bacterial systems are widely used due to the abundance of gene cloning vectors, ease of transformation, and rapid proliferation of cells, but being prokaryotes, they cannot provide the full complement of post-translational modifications found in eukaryotes (reviewed in Rosano and Ceccarelli, 2014[158]). In this study, a eukaryotic expression system was considered more suitable, as NS1 is known to be post-translationally modified and modSP is predicted to have post-translational modifications.

NS1 is a highly conserved flavivirus protein containing six disulphide bonds[159], ranging in molecular weight from 46–55 kDa depending on the extent of glycosylation and is highly immunogenic[160]. N-linked glycosylation of NS1 is important for efficient secretion, virulence, viral replication and hexamer stability[155,161–166]. The dimeric form of NS1 is often associated with

the membrane[167], while the secreted protein is hexameric. It was initially thought NS1 wasn't hexameric when secreted by insects[168], but several reports show both hosts can form the hexameric structure[169–171]. The characteristics of NS1 change as it matures[161,172] and forms a stable dimer which is resistant to SDS[161,168,173].

NS1 is known to activate mammalian Toll-like receptors and to inhibit the complement factors[164,174,136,137,175], presumably in the secreted hexameric form. As this study was examining an extracellular interaction between NS1 and modSP, it was important that an expression system be chosen that would permit hexameric assembly, if possible. ModSP has multiple disulphide bonds and two predicted N-linked glycosylation sites. Glycosylation is typical of many secreted proteins, and variations in glycosylation of the extracellular signaling cascade have been observed to modulate immune signaling in *Drosophila*[176]. While there are no studies that describe the extent of post-translational modifications of this protein, it is anticipated that some post-translational modifications of modSP would also be critical to enable an interaction with the NS1 protein.

The initial choice of human HEK293 cells as the expression system was based on this requirement to produce WNV proteins in a configuration found in a vertebrate host, to maximize the likelihood of producing all variants of the NS1 protein. Difficulties in production of either NS1 and modSP in the HEK293 cells could be attributed to many factors, including poor replication or retention of the expression plasmid, suboptimal transcription, low translation rates, inefficient post-translational modifications, and poor secretion rates. None of these factors were examined fully in this study, as it would take considerable time to examine all of these steps, and if any of them were problematic, resolving the problem could take many months of trouble-

shooting without necessarily producing functional proteins in the end. However, among the aforementioned factors, the issue of secretion was considered solvable by switching to a different *in vivo* expression system, using baculovirus expression vectors in cultured insect cells.

The baculovirus expression system was considered a good choice to maximize protein yield, as the pFastBac vector backbone uses the polyhedrin promoter for high yields of recombinant protein[177]. The plasmid also carries the secretion signal from *Autographa californica* gp67[178,179] to enable secretion of the recombinant proteins into the medium, for easier purification and mimicking the extracellular nature of the proteins being investigated. Using the Sf9 insect cells as the *in vivo* component of the expression system would produce proteins with post-translational modifications typical of an insect. As this study was attempting to identify proteins interacting within an insect, presumably in the mosquito's tissues beyond the gut, an insect cell line could potentially generate the proteins in a form that more closely matched their post-translational modifications within the insect host than did the human HEK293 cells.

The baculovirus expression system did indeed produce more proteins than did the HEK293 cells, but regrettably, the interaction of modSP and NS1 was still not confirmed from these experiments. There are a variety of reasons that may explain the lack of conclusive results, most of which are associated with the proteins not truly resembling the true configuration of the proteins within the mosquito. Firstly, the recombinant proteins still retained some extra amino acids that could alter their structure and function, despite using a SUMO-fusion tag that would remove almost all extraneous amino acids used in the protein purification process. Secondly, the glycosylation patterns of the native proteins in the infected mosquito are not known, and it is unclear whether the Sf9 cells, which are derived from another distantly-related insect species,

would necessarily modify the proteins in the same configuration in the mosquito. It is also unclear as to whether the NS1 formed the correct quaternary structure required to interact with modSP.

It is also entirely possible that the yeast two-hybrid system, which had previously indicated a NS1-modSP interaction, had generated proteins with atypical post-translational modifications, and hence, the observed interaction of modSP and WNV NS1 was, in fact, an artefact of that particular expression system. The interaction of these two proteins in the previous study was indeed intriguing and worthy of exploration, given that NS1 is known to modulate Toll-like signaling in vertebrates, and modSP is considered to be a key modulator of Toll signaling in other insect innate immunity responses. The lack of interaction seen in this current study does not necessarily negate that NS1 interacts with modSP, as it is still unclear if the proteins generated in this expression system reflect their true configuration and interactions within the mosquito. ModSP is typically produced in an inactive zymogen form[91], and in *D. melanogaster,* modSP activation is autocatalytic[91], while in *T. molitor*[180] and more recently, in *An. gambiae*[181] modSP is stable and requires other components of the serine protease cascade to activate it. *M. sexta* HP14 only displays autocatalysis when incubated with PGN directly[182,183]. The cleavage sites correspond when aligned, with *Ae. aegypti* showing high homology (Figure 9). It is therefore possible that the *Ae. aegypti* modSP generated in the Sf9 cells of this study was not in a form that was capable of interaction with NS1. As modSP requires cleavage for activation, if there is an NS1-modSP interaction, it could be between the full-length zymogen or active C-terminus of modSP.

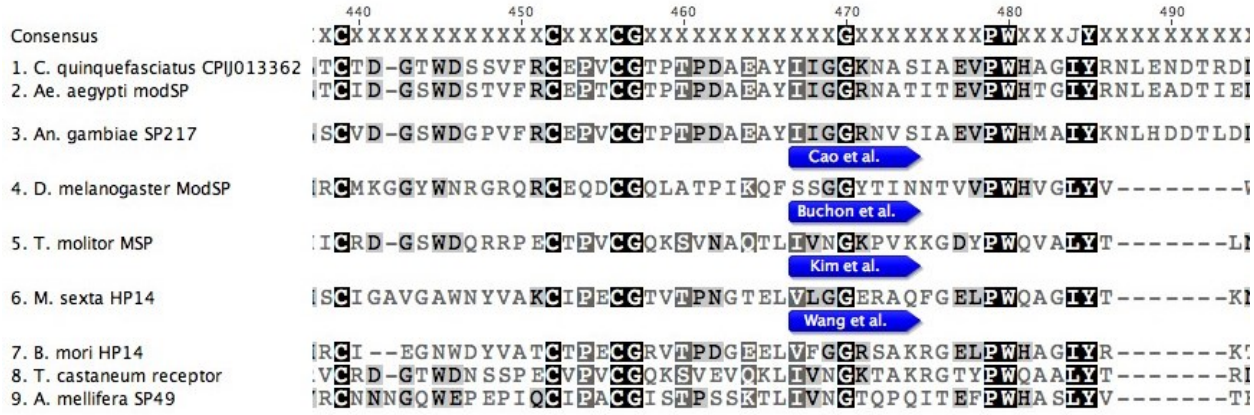


Figure 9. Alignment of modSP homologs from various insects.

To resolve these questions, an expression system in mosquito cells may be more appropriate. At the time that these studies were conducted, baculovirus expression vectors for mosquitoes had not been developed. However, since then, Naik et al.[184] developed a baculovirus transduction system in C6/36 cells, a commonly used *Ae. albopictus* insect cell line with protein production being driven by the mosquito cecropin b1 gene promoter from *Ae. aegypti*. Expression is highest in C6/36 but remains high in *Ae. aegypti* CCL-125 cells. Ultimately, reliable proof would come from *in vivo* interactions in the mosquito with one or both proteins, along with deletion mutant proteins which would be possible using this method. This would determine whether the interaction of these proteins occurs in the mosquito and results in down-modulation of the insect's immune defenses, to allow the WNV to replicate more readily within its mosquito host.

Chapter 3: Identification of innate Immune Responses to a Gram-Positive Bacterium, *Staphylococcus epidermidis*

Introduction

Throughout their lives, mosquitoes are faced with the threat of many different types of infection. During the larval and pupal stages of development, mosquitoes live in an aquatic environment rife with bacteria. As adults, mosquitoes can acquire pathogens through blood feeding, nectar feeding, and through physical injuries. To protect themselves from infection, mosquitoes are capable of mounting multiple innate immune responses that either kill, incapacitate, or engulf the pathogens.

The Toll pathway of innate immunity, which appears to be common to all insects, is typically associated with responding to infections of Gram-positive and fungal pathogens by producing a variety of AMPs that can disrupt the bacterial cell membranes to kill the bacterium, attract hemocytes to either phagocytose the invading cells, or to encapsulate the bacteria with melanin[185]. The Toll pathway has been well characterized in the model insect species *Drosophila melanogaster*, and in the few other insects examined to date, including the beetle *Tenebrio molitor* and the moth *Manduca sexta*, a similar series of events occur to induce the antimicrobial defenses[186]. The pathogen is first detected by pattern recognition receptors (PRRs), which then activate a cascade of serine proteases that ultimately activate Spaetzle, which binds to the Toll receptor. Toll then initiates an intracellular signaling pathway that induces the expression of AMPs. While many of the proteins of the Toll signaling pathway are conserved in the insects examined to date, differences in signaling components have been observed in different species, particularly in the extracellular signaling cascades that regulate Toll induction. A diversity of serine proteases (SPs), such as modSP and CLIP-domain containing-SPs, along with a diversity

of modulating serine protease inhibitors (serpins) have been identified in the various insects that have been examined to date[186].

The Toll pathway has been demonstrated as an important antibacterial defense system in *Ae. aegypti*[93], although in that study, only a few components of the Toll pathway were identified. Innate immunity responses, including the Toll pathway, have also been observed in virus infections in *Ae. aegypti*[187]. The complete diversity of Toll signaling components in this mosquito has not yet been examined following a bacterial infection. The availability of a recently updated *Ae. aegypti* genome, in combination with relatively straightforward methods of evaluating gene functions using RNAi techniques, provides an opportunity to study the mosquito's responses to a bacterial infection. In this study, a transcriptomic analysis using long-read Nanopore sequencing enabled the identification of a suite of genes that are activated during a Gram-positive bacterial challenge. Using RNAi-mediated knockdown, the role of some of the proteins were examined, in an effort to identify those genes that play significant roles in the mosquito's response to a bacterial infection.

Methods

Insect rearing

Ae. aegypti mosquitoes were reared at 28°C with a 16:8 h light:dark cycle with 60% relative humidity. Eggs were hatched in deionized water and larvae were fed ground liver powder and rabbit food pellets. Adults were fed *ad libitum* 10% sucrose water and females were blood-fed once weekly on warmed (42°C) rat blood sealed within two layers of stretched Nescofilm (Karlan Research Products).

Infecting Aedes aegypti mosquitoes

Gram-positive bacteria, *Staphylococcus epidermidis* were grown overnight in liquid LB. The cells were pelleted at 8,000xg and the media was removed. Mosquitoes were cold-anesthetized on ice. A 0.15 mm Austerlitz minutiens Insect Pin (Entomoravia) was dipped in sterile PBS or the bacterial *S. epidermidis* pellet and used to pierce the center of the thorax avoiding the ventral nerve cord and heart. The treated mosquitoes were placed in individual fly vials and fed 10% sucrose from a fresh cotton swab daily. To examine mortality, the mosquitoes were assessed for survival daily for 14 days. The date of death or end date of the experiment was noted with a dead or alive status. Survminer[188] was used to create Kaplan-Meier plots and perform pairwise comparisons with Benjamini-Hochberg correction. The R package coxme[189] was used to perform a mixed effects Cox regression for survival analysis with experiment day as a random variable to examine dsRNA and *S. epidermidis* effects on survival of the mosquitoes.

Transcriptomic analyses of S. epidermidis-infected mosquitoes

Adult female mosquitoes were infected with *S. epidermidis* as described above. At 0, 3, 6, and 24 hours post-infection (hpi) RNA was extracted and purified from three biological replicates of pools of 20 insects as previously described. The mRNA was enriched using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) and the Direct cDNA kit (SQK-DCS108 with native barcoding EXP-NBD103; Oxford Nanopore Technologies). RNA libraries were loaded onto MinION R9.4 flowcells (Oxford Nanopore) and raw reads were generated by MinKNOW. Raw reads were analysed using Guppy 3.0.3 with GPU-accelerated high-accuracy flip-flop base-calling. Quality control on the Nanopore reads was examined by MinIONQC[190] or pycoQC[191] with a Phred quality (Q) score >=7. Pipeline-

transcriptome-de[192] was used to analyse differential gene expression, comparing all four time points (0, 3, 6, and 24 h) to each other. The EnhancedVolcano[193] R package was used to plot genes from the pipeline with a FDR cut-off of 0.05. Genes present in all sample combinations of the filtered-treatments were graphed using ComplexHeatmap in R[194].

Isolation of immune gene fragments from Ae. aegypti

RNA was isolated from 20 *Ae. aegypti* mosquitoes using QIAshredders (Qiagen), the GeneJET RNA Purification Kit (Thermo Fisher Scientific) and cDNA was synthesized using qScript cDNA SuperMix (Quantabio). Fragments to produce dsRNA were PCR-amplified from cDNA using primers listed in Table 1. The PCR products were digested with FastDigest KpnI and XbaI (Thermo Fisher Scientific) or, in the case of modSP ApaI and XbaI, and resolved by electrophoresis. The digested DNA fragments were gel-purified using a QIAquick Gel Extraction Kit (Qiagen) and gene fragments were ligated into a similarly-digested pL4440 dsRNA expression plasmid. The cloned gene fragments' identities were confirmed by DNA sequencing followed by comparisons to the National Center for Biotechnology Information (NCBI) database GenBank[195] and VectorBase[196].

To prepare template DNA for the *in vitro* transcription reactions, the primers pL4440 F (CAACCTGGCTTATCGAA) and pL4440 R (TAAAACGACGGCCAGTGA), were used to amplify the dsRNA-target regions from the pL4440 plasmids. 200 ul of the PCR products were subsequently used as templates to prepare dsRNAs by *in vitro* transcription using a MEGAscript T7 Transcription Kit (Thermo Fisher Scientific).

Table 1. Primers used to create dsRNA constructs cloned into pL4440. Sequences GTATA, GTA and TAA were used to allow cleavage at the ends of the linear PCR product.

Name	Sequence
Ae CLIPB1 KpnI F	GTATAGGTACCTCTGTCTACCGGTGTCGGAGG
Ae CLIPB1 XbaI R	GTATATCTAGACACTCCCGGCACGTCTTCG
Ae CLIPB13A KpnI F	GTATAGGTACCGCCGAAATGGAGGCTGGACC
Ae CLIPB13A XbaI R	GTATATCTAGAGCCTGTCGGAGACCGTTTGG
Ae CLIPB13B KpnI F	GTATAGGTACCACGGCCGACCTCCAAAAGC
Ae CLIPB13B XbaI R	GTATATCTAGAGGCTGTACACGCCAGGAACG
Ae CLIPB26 KpnI F	GTATAGGTACCGATCTGCCTTCCGGTGACGC
Ae CLIPB26 XbaI R	GTATATCTAGAGGTGTAGATGCTGGGCACGC
Ae CLIPB33 KpnI F	GTATAGGTACCTGGTAACGGCTGCCCATTGC
Ae CLIPB33 XbaI R	GTATATCTAGACGACCGGACTCCGTACGACC
Ae CLIPB34 KpnI F	GTATAGGTACCTGCTGTGCATCGTCCAACCC
Ae CLIPB34 XbaI R	GTATATCTAGACTCGACGGCCATGTCAACCG
Ae CLIPB35 KpnI F	GTATAGGTACCTCCACTACCGGAAGCCCAGC
Ae CLIPB35 XbaI R	GTATATCTAGACCCAACCGGCAGCATATCCG
Ae CLIPB5 KpnI F	GTATAGGTACCTCCTGAGCCGGAGTCAGTGC
Ae CLIPB5 XbaI R	GTATATCTAGACTCCAACGCCTTCGCAGTCC
Ae GNBPA1 KpnI F	GTATAGGTACCGATGGGCATCGGTTGCGAGG
Ae GNBPA1 XbaI R	GTATATCTAGACAACGGACCCGTCATGCTCG
Ae GNBPA2 KpnI F	GTATAGGTACCGAACCATGCCGAACCCCTCC
Ae GNBPA2 XbaI R	GTATATCTAGACGTGTGCATCGCCTTGACG
Ae GNBPB1 KpnI F	GTATAGGTACCATTCGTCCCACGCTCACGG
Ae GNBPB1 XbaI R	GTATATCTAGACGTCTGCCCAAACTGACGGG
Ae GNBPB3 KpnI F	GTATAGGTACCGTGCCCCGAAGTGTGTAGCG
Ae GNBPB3 XbaI R	GTATATCTAGATGCCAGCACGTTCACAACCC
Ae GNBPB4 KpnI F	GTATAGGTACCGTGTTGCGGATGGAACCC
Ae GNBPB4 XbaI R	GTATATCTAGATTGTCGCTGGAGGGTCAGGG
Ae GNBPB5 KpnI F	GTATAGGTACCGACGGCGACGTTCACTAGG
Ae GNBPB5 XbaI R	GTATATCTAGATCCACGTGGGCAACCAATCG
Ae GNBPB6 KpnI F	GTATAGGTACCACTGATGCACCGGGTTGGG
Ae GNBPB6 XbaI R	GTATATCTAGATGGGAACTGCGCACATTCGG
Ae modsp dsRNA ApaI F	GTATAGGGCCCTCAGCGGAAGCAGATTACCT
Ae modsp dsRNA XbaI R	GTATATCTAGAAGTGCGCAGCGGATACTACT
Ae modsp newdsRNA KpnI F	GTATGGTACCCACTCCCTCCAACGACCAAA
Ae modsp newdsRNA XbaI R	GTATTCTAGACGTAACGTCCCAGAAGCAGT
Ae spz dsRNA KpnI F	GTATAGGTACCCAGACGACGAGTTTCTTTGCCCC
Ae spz dsRNA XbaI R	GTATATCTAGACACTTACAGCATGATGGTAATCG
Ae SRPN10 KpnI F	GTATAGGTACCGACGCGTTTGGTATTAGTCAATGC
Ae SRPN10 XbaI R	GTATATCTAGAGGTGCCCATTCCAAGCTTTTCC

Name	Sequence
Ae SRPN20 KpnI F	GTATAGGTACCACCGCAGGAACTTCGACC
Ae SRPN20 XbaI R	GTATATCTAGACCGTGATCCGGCAGATTGGC
Ae SRPN21 KpnI F	GTATAGGTACCATTCGGGCAGTAGAGCTGGC
Ae SRPN21 XbaI R	GTATATCTAGATCCGTCCCTACCCGCATCG
Ae SRPN22 KpnI F	GTATAGGTACCTGGGGATGACCATGCTGGC
Ae SRPN22 XbaI R	GTATATCTAGATCCCACTGGCAAGCGAAAGC
eGFP dsRNA KpnI F	TAAGGTACCCGGCCACAAGTTCAGCGTGT
eGFP dsRNA XbaI R	TAATCTAGACTTCGGGCATGGCGGACTT
small gus KpnI F	GTATAGGTACCCCCTTACGCTGAAGAGATGC
small gus XbaI R	GTATATCTAGAGGCACAGCACATCAAAGAGA

Feeding mosquito larvae dsRNA-expressing bacteria

The pL4440 plasmids containing mosquito gene fragments were used to transform HT115(DE3) *E. coli* cells. HT115 cells have the *rnc* gene disrupted by the Tn10 transposon (*rnc-14*::ΔTn*10*)[197], resulting in an RNase III-deficient strain. The strain was also lysogenized with the λDE3 lysogen to add a T7 RNA polymerase gene to the bacterium's genome[198], under the control of a lacUV5 promoter for high levels of production[199].

HT115(DE3) *E. coli* cells were grown in liquid LB cultures supplemented with 50 μg/ml ampicillin and 12.5 μg/ml of tetracycline. To produce dsRNA, cultures were grown in 60 ml of LB with ampicillin and induced at an OD_{600} of 0.4 by the addition of IPTG to a final concentration of 0.4 mM. Once the cultures had reached an OD of 0.7-0.8 after 4 hours, the cells were pelleted by centrifugation, heat-killed at 80°C for 15 minutes, and then mixed 500 ul of a 10% w/v solution of brewer's yeast and suspended in 2 ml of 1% agar. The agar-bacteria mixture was transferred into an open-ended syringe and cooled. The solidified agar was sliced into 0.5 ml discs and stored at 4°C for up to 1 week before use. Mosquito larvae were treated in densities of 1 larva/2.5 ml water (groups of 20 larvae in 50 ml) at room temperature and fed two 0.5 ml discs

of bacteria-containing agar every three days. As pupae developed, they were transferred to individual vials to await eclosion and sex-sorting.

Assessment of dsRNA produced by bacteria

E. coli HT115(DE3) were transformed with the plasmid pL4440-EGFP, which produces a 300 bp dsRNA from two convergent T7 promoters. Three replicate cultures of 50 ml in 2YT broth supplemented with ampicillin were grown at 37°C with shaking. Production of dsRNA was induced by addition of 0.4 mM IPTG, and 1 ml of bacterial cells was harvested at 5 different time intervals (1 to 5 h after addition of IPTG). RNA was extracted using the SingleShot Cell Lysis Kit (Bio-Rad, Hercules). The iTaq Universal SYBR Green One-Step kit (Bio-Rad) was used to prepare the cDNA for qRT-PCR analysis, using primers specific for the EGFP dsRNA sequence F (GCTGACCCTGAAGTTCATCT) and R (AGAAGTCGTGCTGCTTCAT). No-template controls confirmed that no contaminating genomic DNA was present from any of the bacterial isolates. An internal reference gene, rrsA (NC_000913.3:4035531-4037072), was used to normalize the yield of dsRNA, using rrsA-specific primers F (GCGGACCTCATAAAGTGCGT) and R (CACCGTGGCATTCTGATCCA). Serial dilutions of PCR-amplified templates (EGFP or rrsA) were used to generate a standard curve to enable quantification of the dsRNA production.

Quantification of gene expression

S7 has been used by the Whyard lab and other labs as a stable internal reference gene. Recently, the *Ae. aegypti* genome has been sequenced using long-reads with improved gene annotation[200]. A range of commonly used reference genes have been assessed for suitability

across a range of developmental stages in *Ae. aegypti*[201,202]. Based on these studies, additional reference genes, Actin5C (AAEL011197/XM_001655125.2), 40S ribosomal protein S17 (AAEL025999/ XM_001648517.2) and the 60S ribosomal protein L32 (AAEL003396/XM_001656684.2) were used in some analyses (outlined below).

Droplet digital PCR (ddPCR): RNA was extracted and cDNA was synthesized (as described above) from one to two *Ae. aegypti* mosquitoes to assess the extent of RNAi. Given the heightened sensitivity of ddPCR relative to conventional qRT-PCR, cDNA samples were diluted between 10 to 100-fold, such that only 2-10 ng calculated from the original RNA sample was used as cDNA template for each ddPCR reaction. This enabled the quantification of up to 50 genes to be analysed from a single sample. Gene expression was assessed by ddPCR using the Bio-Rad QX200 AutoDG Droplet Digital PCR System and QX200 ddPCR EvaGreen Supermix (Bio-Rad). Primers used to assess the levels of expression of the *Ae. aegypti* genes are listed in Table 2 to evaluate if more than one gene's expression was affected by the delivery of dsRNA to the larvae. The expression of the 18 genes was expressed relative to the geometric means of the three internal standards S17 and L32 ribosomal protein genes and Actin5C, each of which were amplified from all cDNA samples using the species-specific primers when possible otherwise, a single reference gene was used (Table 2). Primers were designed using Primer-BLAST[203] to *Ae. aegypti* (taxid:7159), first having a primer preferentially spanning an exon junction when possible, primers designed for two exons flanking an intron and, designed to one exon if suitable primers couldn't be created

Table 2. Primers used to assess gene expression in *Aedes aegypti* with ddPCR.

Name	Sequence
Ae act5C ddPCR F	ACCGTAAAGCTCACCGATATCAAC
Ae act5C ddPCR R	GCCTTGCACATACCGGATCC
Ae CLIPB1 qPCR F	ATTGCCAAACCGGATGACT
Ae CLIPB1 qPCR R	TCGTTCGTTGATCAGCACTC
Ae CLIPB13A qPCR F	TCGTCCCAAATACAGCAACGA
Ae CLIPB13A qPCR R	TCTGAAGGGCGGATGTCACT
Ae CLIPB13B qPCR F	TCGTCCGAGTTTCATAGCGA
Ae CLIPB13B qPCR R	GCAGATGGGTTGAATGTGATCTTT
Ae CLIPB35 ddPCR F	ACGGAGAGTGGTAGTGCTAG
Ae CLIPB35 ddPCR F	ACGGAGAGTGGTAGTGCTAG
Ae CLIPB35 ddPCR R	ACTCCACCGGCACATAGTTG
Ae CLIPB35 ddPCR R	ACTCCACCGGCACATAGTTG
Ae CLIPB5 qPCR F	CAACTACTTGGACTTTAGCCGAA
Ae CLIPB5 qPCR R	TCTGGCGAGCAATCAACGTC
Ae DefA qPCR F	GCCACCTGTGATCTGCTGAG
Ae DefA qPCR R	GACAGACGCACACCTTCTTG
Ae DefC ddPCR F	TCGCTCTTACGCCAACTCTTTG
Ae DefC ddPCR R	CACCGAACCCACTCAGCAGA
Ae DefD ddPCR R	AAAGTGCACAGATGTCCATTGA
Ae DefD qPCR F	GTGCCTTCGAGCAAATTATACCTA
Ae GNBPA1 ddPCR F	TGATCCTTTCCGGCTGCAAA
Ae GNBPA1 ddPCR R	GGCAGGATGGTGGACAGG
Ae GNBPA2 qPCR F	GCGCAAATCTCCACGTTCAAC
Ae GNBPA2 qPCR R	GCTAGGTTTGAGATACAGTTGTGGA
Ae L32 ddPCR F	CGTGTTGTACTCTGATCAAATAAGC
Ae L32 ddPCR R	CGGACTGATGGCGGATGAAC
Ae modsp both qPCR F	CATCAAGGAATGCGCCGATAG
Ae modsp both qPCR R	TCGGTGTTTGAGCAATTTCCAG
Ae modsp new ddPCR F	GAGCGTGCATTGGTGGCTAT
Ae modsp new ddPCR F	GAGCGTGCATTGGTGGCTAT
Ae modsp new ddPCR R	GTGGTGGATGGGAATGGCTT
Ae modsp new ddPCR R	GTGGTGGATGGGAATGGCTT
Ae modsp old qPCR F	AGCCATTCCCGTTCACTACAC
Ae modsp old qPCR R	TCGAATAATTGGTGCCGAGGT
Ae PGRPLC3 ddPCR F	GAGTTTCACATGGCGGACGA
Ae PGRPLC3 ddPCR R	CGTTGAAGCCTTTAGTGTGAGC
Ae PGRPS1 ddPCR F	AAGCAGAACAAGTGGAGCGA

Name	Sequence
Ae PGRPS1 ddPCR R	ATGCCAGCCAATTCCTTCGT
Ae Rel1A ddPCR F	TGAGCTACACCTTCAGCAACC
Ae Rel1A ddPCR R	TGACCGTATCCAGTCTTGAACG
Ae Rel2 ddPCR F	ATTGCCGTGGAGAGCGATTC
Ae Rel2 ddPCR R	GAAGTGGCGTGTAACCTGCA
Ae S17 ddPCR F	CGAACAAGCGGATCGTCGAAG
Ae S17 ddPCR R	ATGTGTCACGAAACCAGCGATC
Ae S7 junc qPCR F	CCATTGAACACAAGGTCGACAC
Ae S7 junc qPCR R	GTAGGGCTCCGGGAATTCGA
Ae Toll1A qPCR F	AATCTTTCGGCAAACATCTTCGC
Ae Toll1A qPCR R	ATGGCGTGCAGGAAGTTGTC

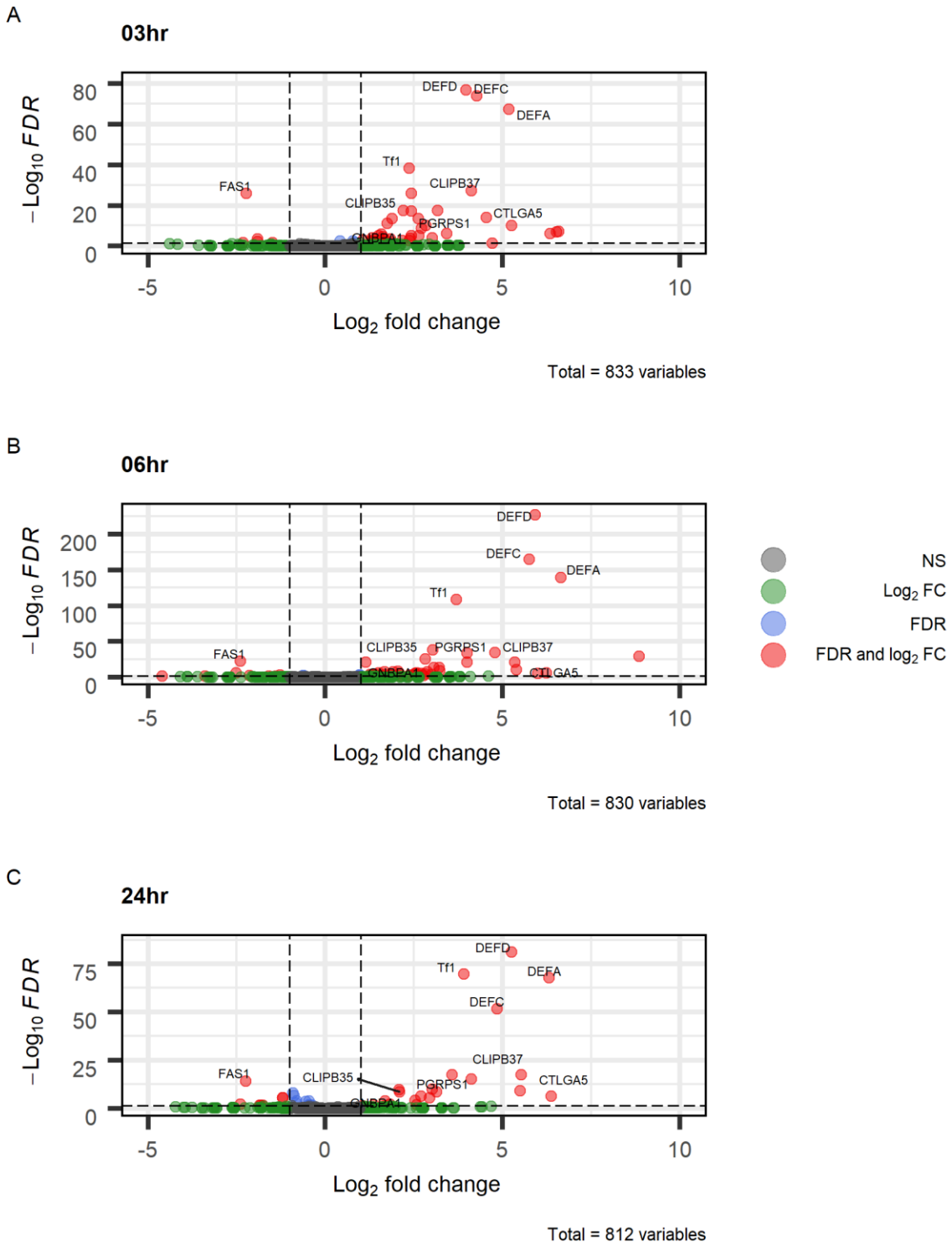


Figure 10. Volcano plots showing the genes corresponding to fold-change of |1| with a FDR of 0.05 at A) 3 hpi, B) 6 hpi, and C) 24 hpi. The data were based on three biological replicates, compared to non-infected 0 h controls.

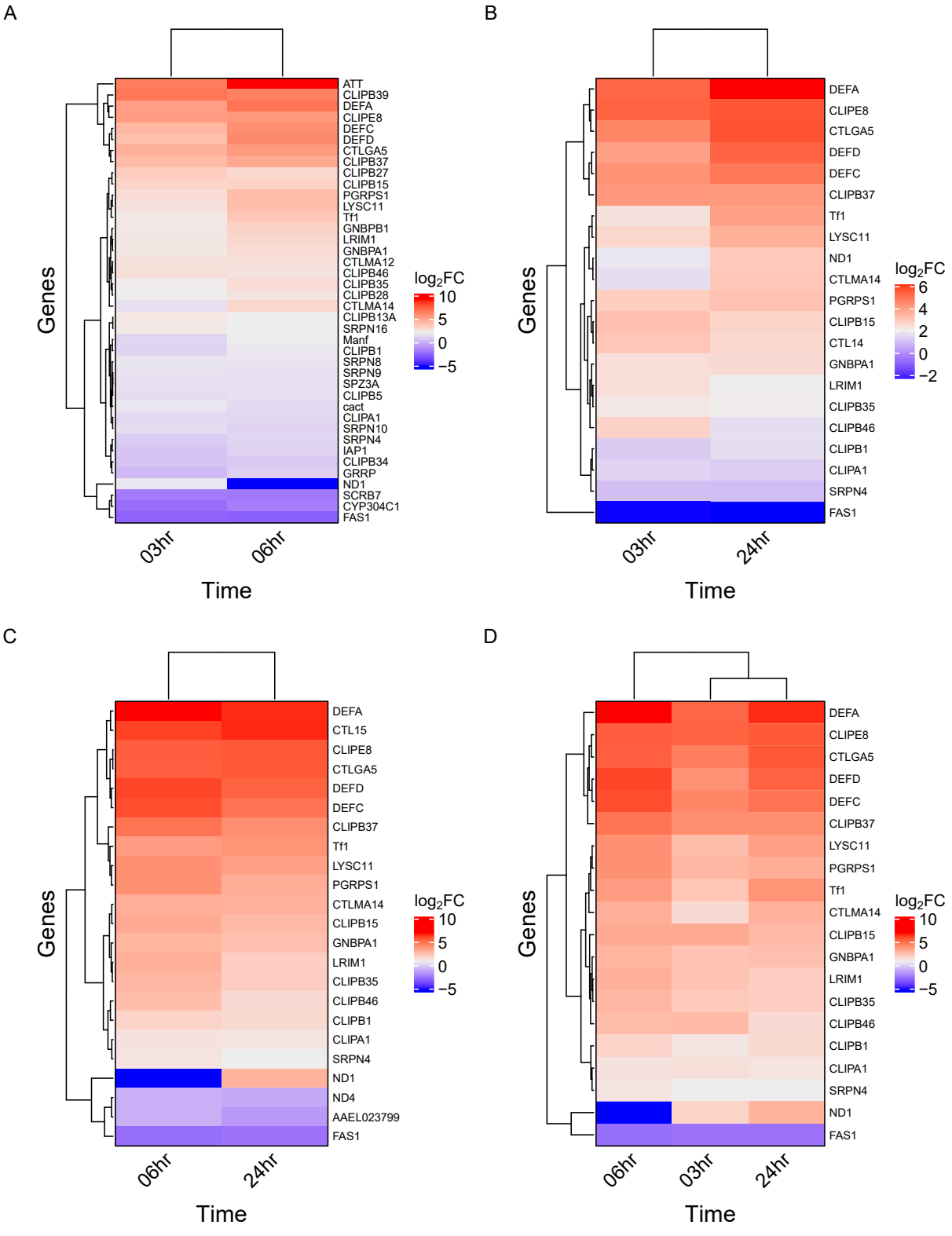


Figure 11. Pairwise or three-way comparison heatmaps illustrating changes in gene expression of three time points (3, 6 and 24 hours) post-infection. Values are expressed as relative changes in transcript abundance relative to uninfected mosquitoes at 0 hours.

Results

Transcriptomic analyses identify genes associated with bacterial infection

Sequencing yielded ~ 3.5 million reads or ~5.5 GB of data with a Q score >=7. The median Q score was 8.5, N50 was 1850 bp with a median length of 1350 bp. Mosquitoes infected with the Gram-positive bacterium *S. epidermidis* were subjected to transcriptomic analyses at 0, 3, 6, and 24 hours post-infection (hpi) to examine which genes exhibited the most significant changes in expression. Infection-responsive genes, relative to the zero-hour time point samples were examined and screened by false discovery rate (FDR). Not unexpectedly, many previously-identified immune response genes showed significant increases in expression 3, 6, and 24 hpi. Volcano plots for each of these time points illustrated that the three defensin genes (defensin A, C, and D) were among a small subset of genes that consistently showed both large increases in transcript abundance (x-axis) and very low FDR scores (y-axis), the latter indicating low likelihood that these changes were false positives (Figure 10). Attacin, which is typically considered an antibacterial peptide active against Gram-negative bacteria, was also strongly up-regulated at 6 hpi. Attacin has however, also been observed to be active against fungi and protozoans[204], and in the case of *Spodoptera exigua*, active against Gram-positive bacteria *Bacillus subtilis* and *Listeria monocytogenes*[205]. In *Ae. aegypti*, attacin has indeed been shown to also be activated by Gram-positive bacteria, but not fungi[119].

A list of the most significantly up- or down-regulated genes was compiled and 43 out of 46 of the up-regulated genes were known or predicted immune response genes (Table 3). In addition to the defensin genes and the one attacin gene, three additional antibacterial peptide genes were also up-regulated, including genes encoding a C-type lysozyme, a leucine-rich

immune protein, and a protein with homology to holotricin, an AMP first characterized in the beetle *Holotrichia diomphalia*[206]. Genes encoding bacterial detection proteins (e.g. PGRPS1, GNBPA1, GNBPB1) and extracellular signaling cascade proteins (e.g. 14 serine proteases and 5 serine protease inhibitors), were also up-regulated. Five different C-type lectin genes were upregulated, which is not surprising, as these cell surface proteins are considered to act as pattern recognition receptors that can mediate encapsulation and melanization of bacterial pathogens[207]. Of the intracellular signaling pathway, the cactus gene was the only gene directly associated with the Toll signaling pathway to be up-regulated. A small number of other genes, not directly associated with immune signaling pathways were up-regulated, including transferrin, mesencephalic astrocyte-derived neurotrophic factor (Manf), 2 NADH dehydrogenase subunits, and an inhibitor of apoptosis. Of all the aforementioned genes, only the three defensin genes, PGRPS1, and GNBPA1 were up-regulated at all three time points examined. For the remaining genes, the up-regulation of transcripts was observed for only some, but not all time points.

Only six genes were strongly down-regulated. Two genes encode NADH dehydrogenase subunits, a key enzyme of the mitochondrion's electron transport chain (ETC). Mitochondrial functions can be altered during immune responses, and inhibition of the ETC can lead to increased reactive oxygen species (ROS), which can assist phagocytes in killing bacteria[208,209]. One of the down-regulated genes (AAEL023799) is annotated as a NADH-ubiquinone oxidoreductase chain 1-like pseudogene. Another down-regulated gene encoded a cytochrome P450 of unknown function, one encoded a scavenger receptor protein associated with mediating endocytosis, and the other encoded FAS1, a gene encoding fatty acid synthase. The necessity of down-regulating these genes during the innate immunity responses is unclear, but worthy of further exploration.

It is worth noting that all of the strongest responding genes identified in this screen showed significant increases (or decreases) by the first time point, indicating that the immune responses occur rapidly, within the first 3 h of infection. For many of the genes, differential expression was observed throughout the time course. A heat map was created for the three time points, to visualize changes in gene expression relative to time zero (non-infected) insects (Figure 11). Figure 11A illustrates the changes in gene expression during the time interval 3 to 6 hpi. By this time point, some genes are starting to show decreases in transcription, while 21 genes continue to show pronounced increases in expression relative to time zero. The PRR genes, for example peaked at 6 hpi, and thereafter show some decline by 24 hpi. Many of the serine protease genes peaked early, at 3 hpi and began to show declines thereafter, and similarly, the effector genes encoding AMPs peaked at 6 hpi and then showed slow declines by 24 hpi. From these analyses, the 6 hpi time point appears to show the greatest shift in gene expression, relative to the other two time points (Figure 11D), suggesting it may represent the peak period of regulation of immune gene expression.

Table 3. Gene fold-change at 3, 6 and, 24 hours post infection compared to 0 hours after immune challenge of *Aedes aegypti* with *Stapylococcus epidermidis*.

Type	Gene	Description	Fold induction (relative to time 0hr)			VectorBase
			03hr	06hr	24hr	
Up-regulated						
Extracellular (Detection of Pathogens)	CTL14	C-Type Lectin (CTL14)	3.02	N/A	2.58	AAEL011453
	CTL15	C-Type Lectin (CTL)	N/A	5.99	6.38	AAEL012353
	CTLGA5	C-Type Lectin (CTL)	4.54	5.35	5.52	AAEL005641
	CTLMA12	C-Type Lectin (CTLMA12)	2.64	2.71	N/A	AAEL011455
	CTLMA14	C-Type Lectin (CTL)	1.68	3.06	3.01	AAEL014382
	GNBPA1	Gram-Negative Binding Protein (GNBP)	2.42	2.88	2.54	AAEL007626
	GNBPB1	Gram-Negative Binding Protein (GNBP)	2.40	3.23	N/A	AAEL003889
	PGRPS1	Peptidoglycan Recognition Protein (Short)	2.84	3.99	3.14	AAEL009474
Extracellular (Signaling)	CLIPA1	Clip-Domain Serine Protease family A	1.51	1.41	1.37	AAEL002601
	CLIPB1	Clip-Domain Serine Protease family B	1.33	1.91	1.69	AAEL000074
	CLIPB5	Clip-Domain Serine Protease family B	1.63	1.69	N/A	AAEL005064
	CLIPB13A	Clip-Domain Serine Protease family B	2.43	2.05	N/A	AAEL003243
	CLIPB15	Clip-Domain Serine Protease family B	3.17	3.21	2.70	AAEL014349
	CLIPB27	Clip-Domain Serine Protease family B	3.43	3.00	N/A	AAEL007993
	CLIPB28	Clip-Domain Serine Protease family B	2.18	2.55	N/A	AAEL013245
	CLIPB34	Clip-Domain Serine Protease family B	0.78	0.98	N/A	AAEL000028
	CLIPB35	Clip-Domain Serine Protease family B	2.21	2.82	2.09	AAEL000037
	CLIPB37	Clip-Domain Serine Protease family B	4.12	4.79	4.11	AAEL005431
	CLIPB39	Clip-Domain Serine Protease family B	6.58	6.24	N/A	AAEL003632
	CLIPB46	Clip-Domain Serine Protease family B	2.72	2.58	1.69	AAEL005093
	CLIPE8	Clip-Domain Serine Protease family E	5.26	5.40	5.50	AAEL005792

Type	Gene	Description	Fold induction (relative to time 0hr)			VectorBase
			03hr	06hr	24hr	
	SPZ3A	spaetzle-like cytokine	1.68	1.63	N/A	AAEL008596
	SRPN10	Serine Protease Inhibitor (serpin)	1.58	1.31	N/A	AAEL007765
	SRPN16	Serine Protease Inhibitor (serpin)	2.36	2.08	N/A	AAEL014138
	SRPN4	Serine Protease Inhibitor (serpin)	1.07	1.39	1.04	AAEL013936
	SRPN8	Serine Protease Inhibitor (serpin)	1.84	1.83	N/A	AAEL011777
	SRPN9	Serine Protease Inhibitor (serpin)	1.75	1.68	N/A	AAEL008364
Immune Effectors	ATT	attacin AMP	6.33	8.85	N/A	AAEL003389
	DEFA	defensin AMP	5.18	6.65	6.31	AAEL003841
	DEFC	defensin AMP	4.28	5.75	4.84	AAEL003832
	DEFD	defensin AMP	3.96	5.92	5.25	AAEL003857
	GRRP	holotricin glycine rich repeat protein (GRRP)	0.42	1.14	N/A	AAEL017536
	LRIM1	leucine-rich immune protein (Long)	2.42	3.04	2.08	AAEL012086
	LYSC11	C-Type Lysozyme (Lys-A)	2.62	4.00	3.57	AAEL003723
Intracellular (Immune signaling)	cact	protein cactus (TOLL pathway signaling)	1.88	1.51	N/A	AAEL000709
Other	IAP1	Inhibitor of Apoptosis (IAP)	0.83	1.26	N/A	AAEL009074
	Manf	arginine-rich protein, putative	1.38	2.09	N/A	AAEL007286
	Tf1	transferrin	2.36	3.69	3.91	AAEL015458
Down-regulated						
	AAEL023799		N/A	-0.63	-1.20	AAEL023799
	CYP304C1	cytochrome P450	-1.91	-1.39	N/A	AAEL014413
	FAS1	fatty acid synthase	-2.23	-2.39	-2.25	AAEL001194
	ND1	NADH dehydrogenase subunit 1	1.88	-4.60	2.95	AAEL018687
	ND4	NADH dehydrogenase subunit 4	N/A	-0.61	-0.82	AAEL018680
	SCRB7	Class B Scavenger Receptor (CD36 domain)	-1.48	-1.60	N/A	AAEL000234

RNAi to explore immune gene function

To validate the innate immunity role of some of the genes from the transcriptomic screen, mosquito larvae were fed *E. coli* that had been transformed with plasmids that should express long (~300) dsRNAs targeting the putative immune genes. Larvae were fed on agar pellets containing the transformed bacteria mixed with brewer's yeast, and once the larvae had eclosed into adults, they were infected with *S. epidermidis* by piercing their cuticle with a pin dipped in the bacteria. Survivorship over a 14-day period was then assessed to determine whether knockdown of the targeted gene's transcripts would result in a more immediate mortality, due to suppression of the immune genes. Negative control mosquitoes were fed bacteria that produced non-mosquito dsRNA targeting the *E. coli* gene encoding β-glucuronidase.

In the initial screen, six genes were tested for RNAi-mediated knockdown: two PRR genes encoding GNBPA1 and GNBPA2; 3 CLIP-SPs encoding CLIPB13A, CLIPB13B, and CLIPB35; and modSP. Of the six dsRNA treatments, only CLIPB35 experienced significantly faster mortality, in the PBS treated controls from experiment 1 when examined post-hoc pairwise with Benjamini-Hochberg adjusted p-values (Figure 12). The lack of mortality in the other dsRNA treatments was somewhat unexpected, as some of those genes had been up-regulated during the immune responses and were presumably important in providing protection from the bacterial infection.

In a Cox proportional hazards model with mixed effects, the model indicates an increased hazard of death from treatment with ClipB35-dsRNA alone and treatments of ClipB35- and GNBPA2-dsRNA interactions with bacteria (Table 4). GNBPA2 and bacteria increases the hazard of death 3x compared to gus, PBS treated mosquitoes. Having ClipB35 dsRNA itself is a

hazard, increasing the hazard of death ~4x in both treatments. As conditions varied on the experimental day, the mixed model attempted to account for these differences, using the date of the experiment as a random effect.

The variable responses of the mosquito larvae to the ingestion of bacteria expressing dsRNA suggested that the bacteria may not be producing consistent amounts of dsRNA. To examine when the maximal dsRNA was produced following IPTG induction, *E. coli* transformed with a reference dsRNA specific to EGFP were grown for different time intervals, harvested and RNA extracted. QRT-PCR was used to measure the amount of dsRNA produced at each time point. DsRNA was detected within 1 h of induction, and the concentration increased for 3 h, but then curiously decreased (Table 5). This decline in dsRNA production was coincident with a decrease in the production of the bacterial rRNA reference transcripts rrsA. This decrease suggests that the bacteria cultures were suffering from either a loss of viable cells, or that cells were no longer metabolically active, resulting in reduction of both the reference gene and dsRNA expression. The level of dsRNA for the later time points (4 and 5 h post-induction) were highly variable, with standard deviations ranging from 57 to 62% of the reported values. While this experiment only evaluated the levels of one particular dsRNA, this level of variation in dsRNA production suggests that all dsRNAs may show similar variable production rates in the bacteria. This variable dsRNA production may account for the lack of observable knockdown of transcripts in some of the dsRNA feeding treatments.

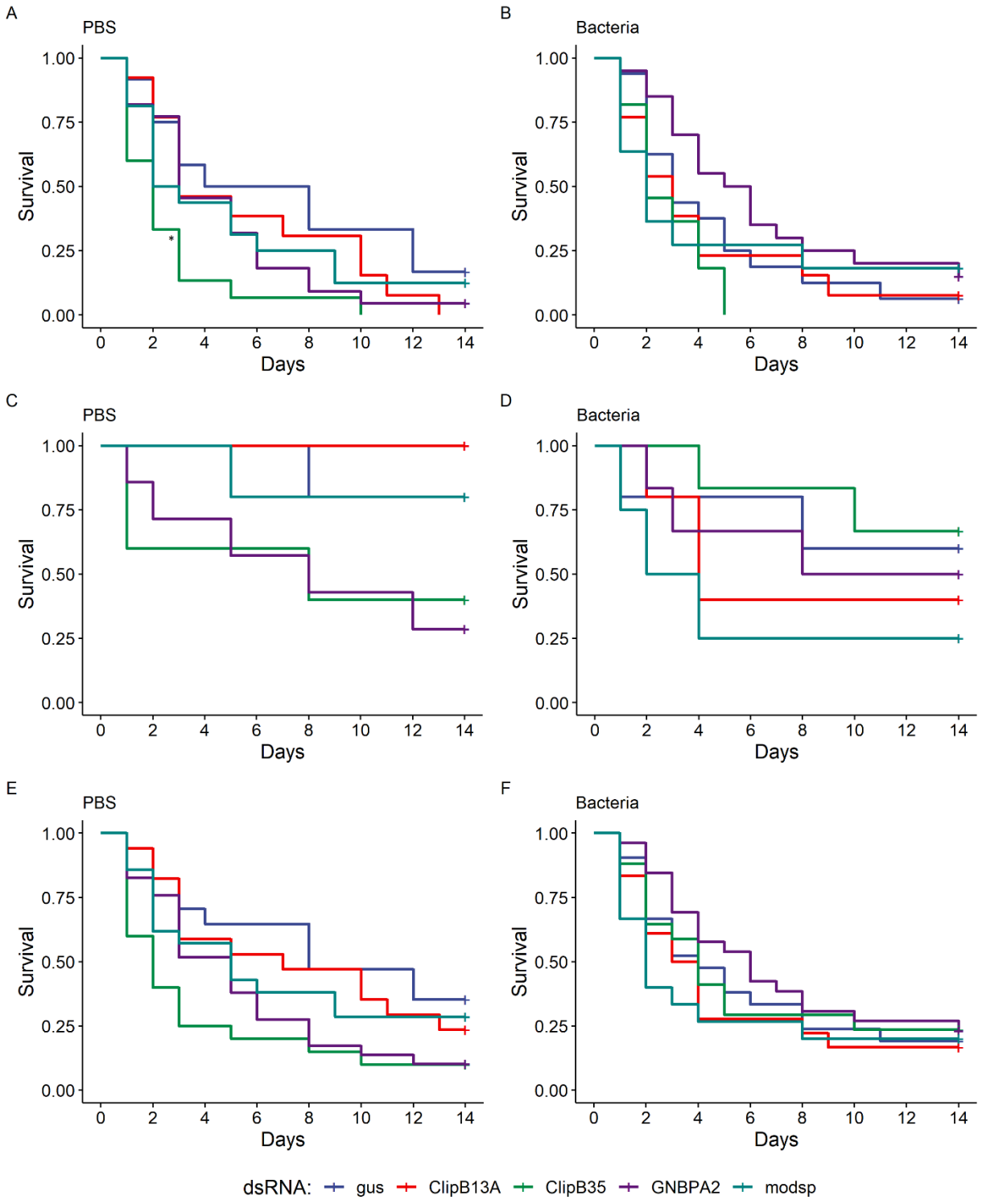


Figure 12. Survival following exposure to *S. epidermidis* in adult, male *Ae. aegypti*. Survival curves corresponding to A) PBS and B) Bacteria for experiment 1. C) PBS and D) Bacteria for experiment 2. E) PBS and F) Bacteria for both experiments grouped. Analysis is examining if different populations have identical survival.

Table 4. Cox proportional hazards model with mixed effects of mosquitoes fed dsRNA and having an immune challenge.

Treatment	coefficient ± SE	Hazard Ratio	p-value
strainClipB13A	0.22 ± 0.41	1.25	0.58
strainClipB13B	0.51 ± 0.42	1.67	0.23
strainClipB35	1.40 ± 0.39	4.06	0.00
strainGNBPA1	0.20 ± 0.38	1.22	0.60
strainGNBPA2	0.70 ± 0.36	2.01	0.05
strainmodsp	0.59 ± 0.39	1.81	0.13
stabtreatmentBacteria	0.70 ± 0.39	2.02	0.07
strainClipB13A:stabtreatmentBacteria	-0.14 ± 0.54	0.87	0.79
strainClipB13B:stabtreatmentBacteria	-0.53 ± 0.58	0.59	0.36
strainClipB35:stabtreatmentBacteria	-1.45 ± 0.55	0.24	0.01
strainGNBPA1:stabtreatmentBacteria	-0.24 ± 0.52	0.79	0.65
strainGNBPA2:stabtreatmentBacteria	-1.12 ± 0.49	0.33	0.02
strainmodsp:stabtreatmentBacteria	-0.39 ± 0.55	0.68	0.48

Table 5. Production of dsRNA from *E. coli* HT115(DE3) transformed with the pL4440 plasmid expressing EGFP-dsRNA. Values represent the means and standard deviations of three biological replicates.

Hours post-induction	[EGFP-dsRNA]*	[rrsA RNA]*	[dsRNA]/[rrsA]
1	2951 ± 199	692 ± 288	4.26
2	4479 ± 1236	955 ± 45	4.69
3	3048 ± 442	1086 ± 181	2.81
4	359 ± 204	489 ± 81	0.7
5	971 ± 608	406 ± 162	2.39

* Concentration expressed as ng/ul of RNA extracted from a 1 ml aliquot of bacteria

The variable levels of dsRNA in the *E. coli* suggests that the mosquitoes may not have acquired effective doses of dsRNA during the entire course of their bacterial feeding. To evaluate whether insects were indeed affected by the dsRNA, RNA was extracted from pools of 20 similarly fed 1-day old mosquitoes, cDNA was prepared, and droplet digital PCR (ddPCR) was used to assess whether the targeted genes' transcripts were reduced. Given the sensitivity of ddPCR, the cDNA samples could be diluted extensively and not just the one targeted gene's transcripts were measured, but 18 other genes' transcription levels were also assessed. Due to the

limited number of mosquitoes surviving the dsRNA feeding treatments, only two biological replicates were performed, for just the two dsRNAs that caused significant mortality (GNBPA2 and CLIPB35) and for mosquitoes treated with modSP, as this gene was of interest in Chapter 2, for its possible role in innate immune responses to WNV infections.

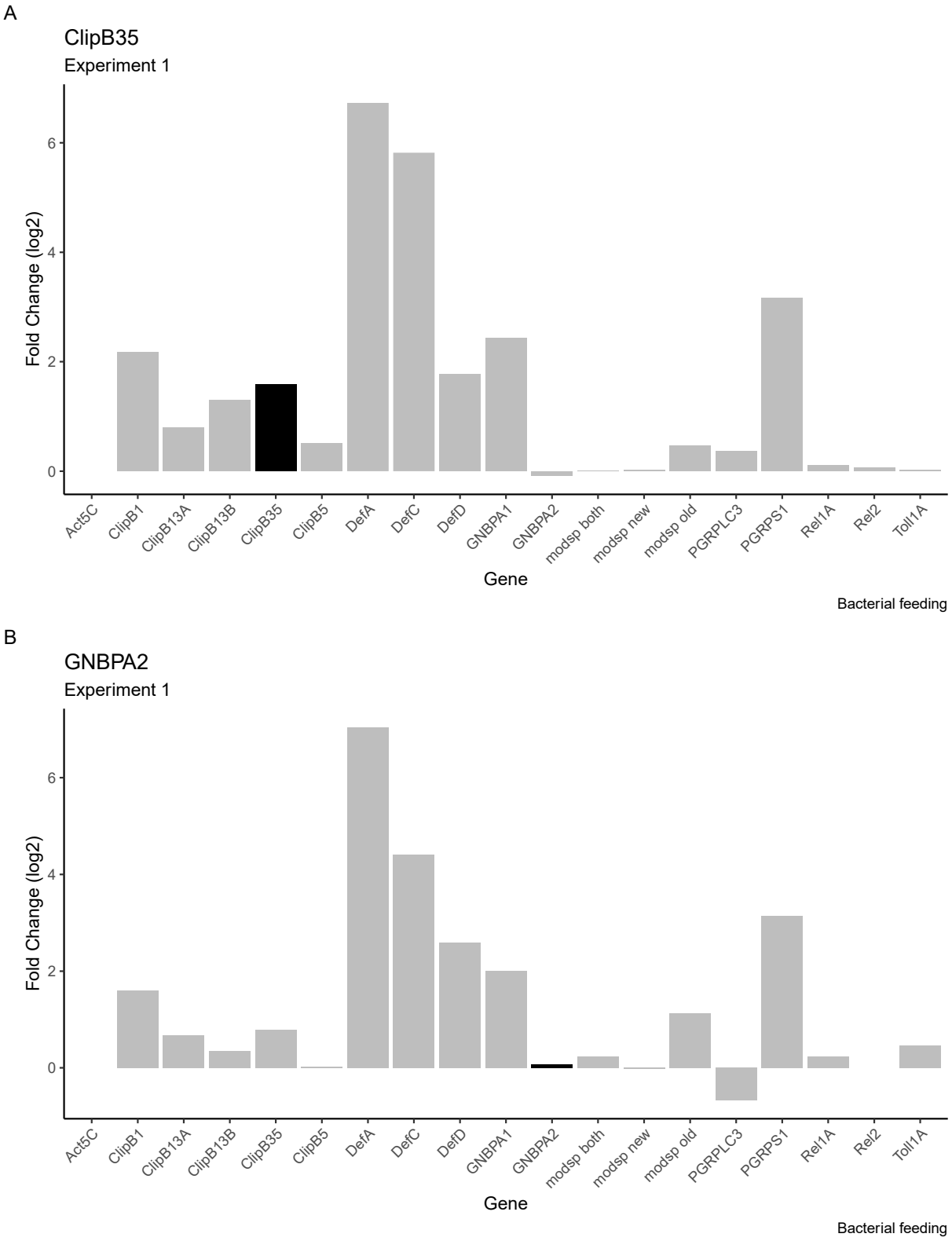


Figure 13. Experiment 1 expression in 2-3-day old post-eclosion adult *Aedes aegypti* after feeding larvae mosquitoes IPTG-induced *E. coli* HT115(DE3) transformed with pL4440 containing gene fragments.

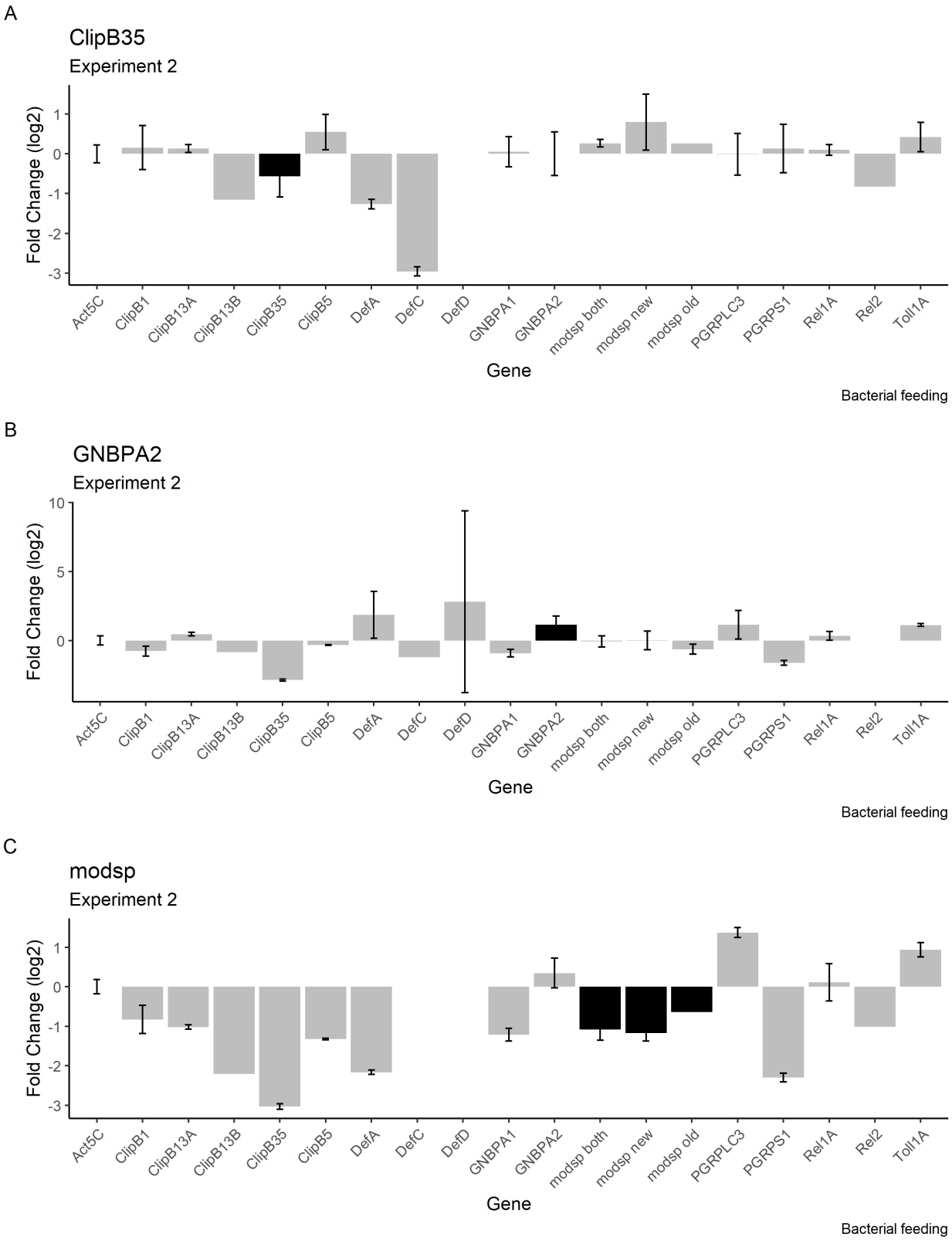


Figure 14. Experiment 2 expression in 2-3-day old post-eclosion adult *Aedes aegypti* after feeding larvae mosquitoes IPTG-induced *E. coli* HT115(DE3) transformed with pL4440 containing gene fragments.

Two independent experiments/trials were conducted to measure changes in transcript abundance in the adults that had fed on the bacteria transformed with the dsRNA expression plasmids. In the first experiment (Figure 13), only two dsRNA treatments were preformed, targeting CLIPB35 and GNBPA2. In this experiment, many of the insects died early, due perhaps to a variety of biotic (opportunistic bacterial growth in the larval water) or abiotic factors (low humidity in the adult vials, evidenced by live insects stuck to the wall of the plastic vials), and hence, their health was already compromised. In this experiment 1, almost all innate immunity genes showed either no change or were upregulated, indicating the dsRNA had not affected the insects as anticipated.

In the second experiment, all three dsRNA were tested (CLIPB35, GNBPA2, and modSP). The mosquitoes fed bacteria expressing CLIPB35-dsRNA showed only a 33% reduction of the targeted transcripts (Figure 14), but due to the large variance of the two replicates, this extent of reduction was not statistically significant.

Interestingly, three other genes' transcripts were, however, greatly reduced. CLIPB13B was reduced approximately 3-fold, relative to the negative control mosquitoes. As this was one of the genes strongly up-regulated in the bacterially-infected mosquitoes (based on the transcriptomic analyses), this perturbation may have contributed to the additional down-regulation of two of the most abundantly expressed AMPs, defensin A and defensin C.

Mosquitoes fed bacteria expressing GNBPA2-dsRNA curiously showed no significant change in GNBPA2 transcripts, but showed a modest knockdown in two other sensor protein transcripts, GNBPA1 and PGRPS1. Treatment with this dsRNA caused indirect yet significant

decreases in CLIPB35. Unlike the treatment with CLIPB35-dsRNA, treatment with GNBPA2-dsRNA caused highly variable responses of two AMP transcripts, defensin A and D, and did not significantly change defensin C transcript levels. The seemingly different responses of the mosquitoes to CLIPB35 and GNBPA2 dsRNAs is curious, as these two dsRNA treatments caused the only significant mortalities in the viability bioassays.

Mosquitoes fed bacteria expressing modSP-dsRNA showed small, but significant decreases in the targeted transcripts. In the recent re-annotation of the *Ae. aegypti* genome (Release 101), the modSP gene sequence was modified slightly, and hence, three different modSP primer sets were used. The "old" primers were designed to the earlier annotated database, "new" primers were designed to a slightly modified sequence in the most recent database, and "both" referring to primers that amplified a portion of the gene unchanged in both annotations. All three sets provided similar results, namely, all three detected small decreases in modSP transcripts in insects fed the bacteria expressing dsRNA designed to the "old" version of the gene. In addition to the slight reduction in modSP, the same two CLIPs (B13B and B35) were reduced, defensin A was reduced. Defensin C and D could not be detected in some samples, but this lack of detection may be due to a lower dilution used and cannot be used as evidence of complete elimination of the defensins' transcripts. GNBPA1 was slightly decreased, but GNBAP2 was not significantly changed. Overall, this particular dsRNA treatment, while having only a small effect on its target gene, appeared to knock down somewhat more of the other genes examined in this screen.

Discussion

In this study, a transcriptomic analysis of mosquitoes challenged with *S. epidermidis* identified a large suite of immune-responsive genes, many of which have previously been associated with Gram-positive bacterial infections. The responses were rapid, with all of the identified genes showing increased expression within 3 hpi, and many genes continued elevated expression for 24 hpi. From this study, it is clear that many components of the Toll signaling pathway were up-regulated, including increased transcription of genes encoding extracellular receptor proteins (e.g. GNBPs), extracellular signaling components (serine proteases and serpins), intracellular signaling (e.g. cactus), and most strongly, AMPs.

It was intriguing to see that virtually all stages of the pathway showed positive responses to the infection, including the extracellular detection and signaling components. In this study, the induction of protein synthesis was not assessed, but presumably many of the extracellular proteins would be produced and secreted, which could help nearby cells mount their own rapid immune responses. The cells in which responses were already initiated may also sustain their immune responses for extended durations as a consequence of the enhanced detection system. From these preliminary results, it appears that 6 hpi was a peak time point for many of the genes identified, with some subsidence of the responses by 24 hpi. The production of AMP transcripts continued to rise up to the last time point assessed (24 hpi), so further time points would be worth examining to determine how much longer the expression of these proteins would continue. Earlier time points would also be interesting to examine, to assess which genes are the first to respond during the early phases of infection.

A diversity of CLIP serine proteases and serpins were upregulated during the infection, but their precise roles and positions along the signaling cascade have not been established. A comparison of the extracellular cascade of serine proteases in different insects revealed that there can be differences between species in the proteins involved in the cascade that activates Toll[210]. The reasons for these differences across species have not been fully explored. An absence of a protein in one species, may, for example, simply reflect an inability to detect some low abundance transcripts or proteins in some species. Transcriptomic techniques have improved immensely in recent years, and long read sequencing, as was used in this study, could greatly improve our ability to identify rare transcripts, including alternatively spliced variants. While that was not the focus of this study, the identification of alternatively spliced transcripts could produce a broader diversity of regulatory proteins, to modulate the immune responses even more than what others have uncovered thus far. Differences in immune response proteins in different insects could also reflect gains and losses of proteins to provide species-specific adaptive responses to different pathogens.

While there were many genes identified in the transcriptomic analysis worthy of further examination, a primary focus of this study was to evaluate which of the newly identified serine proteases may work in concert with modSP as modulators of the Toll signaling pathway. Using RNAi-mediated knockdown of several of the new CLIPs identified, it was anticipated that their role in enhancing immunity could be determined in bacterial infection bioassays. This goal, however, proved more challenging than first anticipated, and hence, only a small subset of those genes were analysed using this approach.

In initial experiments, mosquitoes were injected with dsRNAs before being challenged by bacteria with a second injection. An advantage of this approach is that each insect can receive equal doses of dsRNA, using an Eppendorf FemtoJet microinjector that can deliver nanoliter volumes. This method of validating immune genes in mosquitoes has proven effective in other studies[211]. However, in this study, the dsRNA injections resulted in high mortalities of insects, as they succumbed to infections even before they were challenged by *S. epidermidis*. Consequently, an oral delivery method, using bacteria transformed with plasmids expressing dsRNAs was chosen, as similar approaches have been used in other RNAi studies in mosquitoes[212,213]. Ensuring that the insects acquired sufficient and equal dsRNA doses to persist to adulthood to cause adequate knockdown proved to be another challenge. In the group-feeding assays, some larvae undoubtedly consumed more food, as differences in the size of the larvae was observed. As the bacteria was mixed with yeast, some may have ingested more yeast than bacteria, thereby providing different dsRNA doses to the mosquitoes. As noted from the analyses of dsRNA production in the bacteria, the bacteria may not produce dsRNA consistently, and hence, some insects may have ingested bacteria with different doses of dsRNA. Optimizing bacterial culturing methods, to harvest bacteria at peak dsRNA production may help minimize this variable. Using bacteria as the dsRNA production system could also have proven problematic in this study, as feeding the insects with a bacterium could induce a premature immune response, even before they were challenged with *S. epidermidis*. If this occurred, it could complicate the analysis of the insects' responses to the second bacterium.

As a consequence of one or more of these variables, the knockdown of the targeted genes was found to be highly variable. For 2 of the 3 dsRNAs tested for gene knockdown using ddPCR, the dose appeared insufficient to induce effective knockdown of the targeted gene.

Persistence of dsRNA from the larval feeding stage to the adult has been observed in other studies[214–216], but in this study, the limited knockdown of the target genes suggests that the dose in the adults was not highly effective. In other studies, knockdowns of 60-100% have been observed[215,216]. The sensitivity of the target gene to RNAi can also be an important factor in causing variability of RNAi responses. Highly expressed genes are often considered difficult to knock down, but little is known of what level of transcripts makes an ideal target gene. As observed in the transcriptomic analyses, all of the genes were induced during the infection process, so it is likely that high doses of dsRNA would be required to knock down these genes that are in the process of up-regulating their expression.

Despite only achieving partial or virtually no detectable knockdown of the three target genes GNBPA2, CLIPB35, and modSP, other immune genes' expression levels were perturbed. These changes in non-target genes could be due to transient knockdown of the target genes having lasting impact on other genes in the pathway. The knockdown of some of these other immune genes, particularly the effector defensins, may account for the bacterial infections having more pronounced impact on those insects treated with those dsRNAs. These findings will require further replication to gain statistical rigour, but they do suggest that RNAi knockdowns can have add-on consequences to genes linked to the targeted genes in gene expression cascades, such as immune responses.

An alternative to bacterial delivery of dsRNA is direct feeding of naked dsRNAs[214,217]. DsRNA soaking has been shown to induce systemic RNAi in various insects[218,219], including mosquitoes[213,215,220–222], but this mode of dsRNA delivery is also prone to variability as a result of gut nucleases that can degrade dsRNA molecules before they reach the target tissues[216,223–225].

Various research groups have found that co-delivery of dsRNAs that target nucleases can, over a surprising short time (2-3 days), reduce the nuclease activity and increase the efficacy of RNAi in insects[216,226,227]. In future studies, it would be worthwhile treating mosquitoes with nuclease-specific dsRNAs to enhance the efficacy of the immune gene dsRNAs considered in this study.

In this study, only one gene was targeted at a time, but RNAi could easily be used to target multiple genes, simply by co-delivery of two or more different dsRNAs. It is likely that knockdown of some genes in the immune pathway may not have a large impact, particularly as there may be redundancy of some components, ensuring that loss of one gene will not seriously compromise the insect's defenses. In future studies, multiple dsRNAs, targeting several genes in a pathway could be simultaneously delivered, and if an effect is observed, the number could be reduced to identify gene interactions.

While this study has only just begun to uncover the complexity of genes involved in anti-bacterial immune responses in the mosquito *Ae. aegypti*, it highlights that there are many components that have not been characterized. These differences may reflect this mosquito's unique adaptations to potential pathogens, but will also help identify conserved components of the pathway in other insects, to understand the origins of these molecular defense systems.

Chapter 4: Conclusions

We often think of mosquitoes as disease vectors, but it is important to consider that they too are threatened by infections of many different microorganisms. A mosquito's exoskeleton provides a barrier to most microbes, but through accidental breaks, the insect can succumb to infection if the innate immunity defenses cannot control the infection. Hemocytes are the primary innate immunity agents, providing a cellular-based component of defense by phagocytosing the pathogens, and a humoral-based component that uses pattern-recognition receptors (PRRs), antimicrobial peptides (AMPs), and a phenol oxidase cascade, to either kill or encapsulate invasive pathogens. While the basic components of the innate immunity systems have been identified, understanding how the responses are modulated could prove invaluable, both from a basic science side, to know more of how immune systems evolved in different species, and from an applied perspective, by finding ways to reduce mosquitoes' disease vectoring capacity.

This study examined mosquito innate immunity responses to two pathogens, one of which, *Staphylococcus epidermidis*, can be pathogenic to the insect[228], and the other, West Nile virus, is considered relatively benign for the insect[229], but poses a threat to the animals that the mosquitoes feed upon.

S. epidermidis is commensal bacteria on humans, can cause infection[230], also can be attractive to *An. gambiae*[231] and *Ae. aegypti*[232]. Infection starts slower but a high number leads to pathogenicity[228]. *Ae. aegypti* show higher robustness in surviving a bacterial infection compared to *An. gambiae*, which could be due to increased AMP induction[233].

The two aspects of this thesis examined the interactions on very different scales. In the study focused on the bacterial infection, the scope of immune responses was viewed broadly, using a transcriptomic approach to identify a large suite of genes that responded to the infection. The study confirmed that *S. epidermidis* induces some expected responses also seen in other insects' responses to Gram-positive bacteria, but provided some insights into which genes are induced within just 3 h, and which genes persist for up to 24 h post infection. Some previously uncharacterized genes were identified, revealing that mosquitoes have a rather broad range of serine proteases and serpins that could be involved in modulating the Toll receptor's induced production of AMPs. The precise roles of many of these genes have not been resolved, but the development of some RNAi-based tools to assess the function of innate immunity genes was advanced. The use of bacteria as vehicles for dsRNA was demonstrated, although such vectors could be problematic for analysing immune responses to other bacteria, if the delivery system was also initiating immune responses of its own. Alternative dsRNA vectors, such as yeast[234,235] or algae[236,237] have been developed for various arthropods, and could prove effective for future studies involved in examining innate immunity in mosquitoes.

In contrast to the broad focus approach of the bacterial study, the study that focused on WNV examined just a single protein-protein interaction, with the aim to determine if the virus had the capacity to down-regulate the insect's immune system. Despite having a very narrow focus, this study presented considerably more technical challenges. Two protein expression systems were tested, but only the baculovirus expression system proved effective in producing sufficient proteins for analysis with little consumables. A clear interaction of WNV's NS1 and the mosquito's modSP was not established, but as the structural integrity of the proteins under study was not fully resolved, there is still some uncertainty that no interaction occurs between

these two proteins. Further tests, preferably using mosquitoes rather than cell cultures, could provide the most definitive answer. Resolving this question will provide new insights into how the virus has adapted to two very different hosts, and may help in the development of therapeutics to either suppress viral replication in humans and in mosquitoes.

This research enabled the development of some innovative approaches to explore the complexities of innate immunity in mosquitoes. Transcriptomic analyses can yield large data sets, which can be challenging to identify biologically relevant targets. In this study, MinION devices (kindly donated by Genome Prairie) enabled long-read nanopore-based sequencing, without out-sourcing the sequencing to an external service. While this in-house process was relatively inexpensive (<$1000 for the 12 sequencing runs completed), the base-calling and annotation of the data demanded some lengthy computing time. Despite the challenges, the relatively low cost of this sequencing method, coupled with its remarkable ability to sequence full-length transcripts should prove invaluable in examining further innate immune responses in this mosquito. Comparisons of other mosquitoes challenged with the same pathogens could illuminate species-specific adaptations and the evolution of the signaling cascades, which currently are poorly understood. Likewise, comparisons of immune responses to different pathogens could provide insights into the versatility of the immune system of mosquitoes.

A second research tool developed for this project was the application of ddPCR to measure changes in gene expression in mosquitoes treated with dsRNA. Most studies using RNAi as a gene validation tool focus only on assessing knockdown of the targeted gene[238]. This highly sensitive method of detecting multiple transcripts from very small samples revealed that the expression of non-target genes, particularly genes in related biochemical or physiological

pathways, could also be affected by a single dsRNA. Using this technology could enable future studies to examine the expression of larger suites of genes during immunity responses, to define gene interactions and resolve how the immune system is modulated.

With the rapid development of new molecular biology tools, it will continue to get easier to conduct larger screens to identify new target molecules for therapeutic and biotechnology applications. DsRNA-based pesticides are being developed by biotechnology industries, with the goal to use these molecules, with their inherent sequence specificity, to target genes specific for pest species[239,240]. In this study, a number of genes, when targeted by dsRNA, adversely affected their survival when they were simultaneously infected with a bacterium. One possible application of these findings could involve the development of pesticidal formulations of dsRNA that suppress the insect's immune defenses, coupled with an otherwise benign, ubiquitous microorganism. Alternatively, and perhaps a little more on the speculative side than entirely practical, it may be possible to enhance an insect's resistance to a viral (e.g. dengue, WNV) or protozoan (malaria) pathogen by treating wild populations with dsRNA formulations that enhance their immune responses, by suppressing negative-regulators of immune responses.

The study of immunity in insects has helped us understand many aspects of animals' immune responses, and with new research tools available, many more useful insights and possible applications should follow.

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