Microbial and restriction factor-mediated modulation of *Aedes Aegypti* vector competence for dengue virus

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Abstract

Dengue virus (DENV) is a single stranded RNA virus principally transmitted by the *Aedes aegypti* mosquito. There is currently no widely used vaccine or cure available for those infected with dengue virus. Vector control methods remain the gold standard strategy for blocking disease transmission. Unfortunately, none of the environmental, chemical or biological mosquito control methods that are currently being employed in endemic regions have had success in curtailing the breadth of dengue transmission. As such, novel dengue control strategies are in urgent need. To begin to address this issue, we have focused on approaches to block the pathogen in the mosquito, and for this a thorough understanding of how dengue virus infection is modulated in the mosquito is required. Studies have shown that complex interactions between the mosquito’s innate immune response, its microbiota and the ingested pathogens are critical determinants of the outcome of pathogen infection. During dengue virus extrinsic incubation period, viral replication in the mosquito midgut results in the activation of immune signaling pathways that produce the mosquito’s antiviral effectors. In our previous studies, we isolated two dengue virus restriction factors (DVRF), DVRF1 and DVRF2, which represent putative downstream effectors of the JAK-STAT immune signaling pathway, and have been proven to exert anti-dengue activity. However, little is known about how these putative effector molecules mediate their anti-dengue effect. We have begun to address this question by elucidating the extent to which DVRF1 and DVRF2 are implicated in the defense against bacterial infections.
As previously mentioned, the interaction between the mosquito’s innate immune response, its microbiota and the ingested pathogens are critical determinants of the outcome of pathogen infection. So, understanding how the microbial component influences mosquito-pathogen interactions is critical. We have shown that a field-derived fungus increases the susceptibility of the *Aedes aegypti* mosquitoes to dengue virus infection. We have suggested a mechanism through which this effect is being mediated, and have also initiated an effort of screening additional mosquito- gut associated fungi for their effects on dengue virus susceptibility. A deeper understanding of the factors that influence the resistance of *Aedes aegypti* mosquitoes to dengue virus infections could possibly result in new strategies to control disease transmission.
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Chapter 1: Introduction

1.1 Dengue virus and its impact worldwide

Dengue fever is a mosquito transmitted viral infection that affects about 390 million people each year (WHO, 2017). The dengue virus itself is a positive-stranded RNA virus of the Flaviviridae family, which causes infection via one of four genetically related but antigenically distinct sub-serotypes (DENV 1 - 4). Antigenic diversity observed during DENV infections results in a lack of cross immunity between the four DENV serotypes, however, an infection by one serotype provides protective immunity against subsequent infections by that same serotype.

Clinically, dengue can manifest itself via a wide spectrum of disease patterns. But infected individuals largely fall within three major categories. There are those who suffer from dengue hemorrhagic fever (DHF), the most severe version of the disease, characterized by plasma leakage and severe bleeding; those individuals who suffer from dengue fever which is a milder form of the disease, characterized by sudden fever, aches and joint pain and lastly, there are those who are carriers of dengue virus but show little to no clinical signs or symptoms of the disease.

Interestingly, individuals who seem to have no apparent clinical manifestation of dengue virus infections, commonly called asymptomatics, are considered to be dead-end hosts for dengue transmission. However, a recent study published in PNAS (Duong et. al, 2015) showed that asymptomatic carriers of dengue virus may significantly contribute towards the transmission of dengue, despite lower that average levels of viremia. This standalone study, if proven to be true, has the potential to greatly improve our
understanding of the global epidemiology of dengue virus, which will in turn change the current paradigm for dengue control.

Over the last few decades, we have seen a dramatic increase in the global incidence of dengue virus infections; not only are more people being infected with the virus in areas previously known to be endemic for dengue, but the global prevalence of the disease is also increasing at an alarming rate. (Figure 1.1.1)

**Figure 1.1.1: The global distribution of dengue fever.**

![Distribution of Dengue fever - caused by the Aedes mosquito](image)

*Source: WHO (2013)*

**Figure 1.1.1: Distribution of Dengue Fever caused by the Aedes mosquito.** From WHO (2013). *Aedes aegypti* mosquitoes are vectors of dengue virus. Dark colored regions depict distribution of dengue fever across the globe.
Estimates obtained from the WHO suggest that there are now over 120 countries currently endemic for dengue virus (WHO, 2016). Other studies, have also estimated that there are upwards of 390 million new dengue infections per year worldwide (Bhatt et. al, 2013). This high incidence rate results in about 500,000 people suffering from severe illness per year, of which 22,000 die (CDC, 2014). This situation has prompted many public health officials to consider dengue to be one of the most pressing public health issues in the world.

The impact of dengue extends beyond the public health sector. With more than half of the world’s population currently at risk of dengue virus infection (Beatty et al, 2008), the social and economic impact of the disease has become enormous, especially in highly endemic regions. Although there is no comprehensive data that shows the actual costs incurred from research and implementation of dengue preventative and treatment strategies, we can easily estimate that millions of dollars are currently being invested into the development of preventive as well as therapeutic strategies to control the disease. Unfortunately, despite all these efforts, most of these approaches have been proven to be largely unsuccessful.

The first dengue vaccine, Dengvaxia (CYD-TDV) by Sanofi Pasteur, cost close to $1.5 billion and took 20 years to develop. CYD-TDV is a live recombinant tetravalent dengue vaccine that has been evaluated as a 3-dose series in regions that are highly dengue endemic. This vaccine, though having acquired licensure in about six countries to date, is by no means a full proof strategy against the dengue epidemic. In fact, data from the clinical trial suggested that the vaccine itself may increases the risk of young dengue-naive recipients developing severe disease via a phenomenon known as immune
enhancement. As such, the WHO has recommended that the vaccine be used only in individuals between the ages of 9-45, in regions with extremely high dengue endemicity.

The existence of multiple antigenically distinct dengue serotypes has proven to be the greatest challenge against the generation of an effective tetravalent vaccine that will provide long-term protection against all DENV serotypes. Despite this, in the last decade or so, the overall vaccine development process has made significant progress. The current dengue vaccine pipeline is far more advanced and more diverse than it has ever been. As of 2017, there are approximately five vaccine candidates currently under evaluation in clinical trials (WHO, 2017).

### 1.2 Dengue transmission by Aedes aegypti mosquito

The two most important mosquitoes responsible for dengue transmission worldwide are the *Aedes aegypti* and the *Aedes albopictus* mosquitoes, representing the primary and secondary vectors of dengue virus, respectively. In Africa, *Aedes africanus* and *Aedes luteocephalus* can also act as potential vectors of dengue. However, because of the highly anthropophilic and peridomestic nature of the *Aedes aegypti* mosquito, we focused our studies on this particular species of *Aedes* moving forward.

There are a few different hypotheses addressing the origin of the *Aedes aegypti* mosquito. However, the most accepted theory postulates that the ancestors of the most domestic form of this mosquito originated from sub-Saharan Africa. *Aedes* has managed to spread throughout all tropical and sub-tropical regions of the world. And this
widespread distribution of *Aedes* has lead to considerable variations amongst different mosquito populations with regards to ecology, behavior, and appearance (McBride et. al).

The *Aedes aegypti* mosquito exists as two subspecies: *Ae. aegypti aegypti* and *Ae. aegypti formosus*, which were previously distinguished by morphology and behavioral characteristics such as feeding preference (Mattingly and McClelland). However, these distinctions have now been abandoned, as they do not correlate with genetic markers for *Aedes* mosquitoes that have been collected and studied from West Africa, (McBride et al, 2014).

Vector competence studies on *Aedes aegypti* collected from West Africa have shown that these mosquitoes are more refractory for dengue virus (Bosio et.al, 1998) when compared to *Aedes aegypti* collected from other geographic locations. Evidently, dengue virus transmission is primarily governed by mosquito strain and virus genotype-specific interactions (Lambrechts, 2009). In addition to both host and viral genetic factors, there may be a host of environmental factors that also influence *Aedes* susceptibility to dengue virus infections.
1.3 Linking dengue transmission to mosquito reproduction

Adult female mosquitoes require a blood meal to lay eggs. Females may take more than one blood meal during their entire life span, and it is these additional blood meals that provide additional opportunities for pathogen transmission.

At about 48-72 hours post-blood meal, gravid females will singly oviposit a batch of about 100 eggs just above the water line or on the inner wet walls of any container filled with water (CDC, 2016). These eggs unlike *anopheles* eggs are resistant to desiccation, and can persist dry for weeks while still remaining viable.

The majority of eggs exposed to moisture will eclose within 3 days of oviposition, although temperature variability can also contribute to hatching times, ranging from 5-8 days (Day, 2016). Larvae that emerge from these hatched eggs transit through 4 developmental stages (L1, L2, L3, L4) that ranges from 5-14 days (CDC, 2016). After the L4 stage, larvae will pupate and undergo metamorphosis into adults.

Adult males typically emerge first and swarm above the larval habitat until females emerge at which point mating commences. Although mosquitoes may survive for up to two weeks in the field, adult male mosquitoes typically die off earlier than female mosquitoes (CDC, 2015).
For a depiction of the mosquito life cycle, see Figure 1.3.1 (Charlesworth 2014).

Figure 1.3.1: The mosquito life cycle. Adapted from Scott Charlesworth, Purdue University (2014). Although the life cycle is generalized, it is applicable to all *Aedes* mosquitoes.
The success of dengue virus lies in its ability to co-evolve with the mosquito vector. By exploiting the anautogenous nature of adult female mosquitoes, transmission of the virus is continuously maintained.

The ability of the *Aedes* mosquito to vector dengue virus is modulated by intrinsic genetic factors. For an infected mosquito to successfully transmit dengue to a host, the virus must first complete its infection cycle in the mosquito.

First, DENV must overcome a midgut infection barrier (MIB), which prevents ingested virions from entering and replicating in mesenteronal cells. Once this barrier is overcome, the virus begins to spread laterally to neighboring epithelial cells to successfully establish infection in the mosquito midgut. After successful replication in the midgut epithelium, the virus must pass through the midgut escape barrier (MEB), which prevents virions from escaping from the basal lamina of midgut cells and disseminating to other tissues in the hemocoel. DENV can then begin its replication in other tissue such as Malpighian tubules, fat body, trachea and the salivary glands. The mechanism(s) by which DENV disseminates from the midgut are not well understood. However, during the course of the infection, DENV must successfully replicate and diseminate into the hemocoel, and finally infect the salivary glands to be shed in the saliva for transmission to the next vertebrate host. Once a mosquito is productively infected with dengue virus, it is capable of transmitting the virus for life.
For a depiction of the mosquito infection cycle, see Figure 1.3.2 (Purdue University, 2014)

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**Figure 1.3.2: The mosquito infection cycle.** Adapted from Scott Charlesworth, Purdue University (2014). Although the infection cycle is generalized, it is applicable to all dengue virus infections, the blue dots representing the journey of the virus from ingestion during a blood meal to transmission during a subsequent blood meal.

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1.4 *The mosquito’s immune system*

Insects are constantly being exposed to substantial selective pressure from pathogens in their environments, thereby significantly influencing the evolution of insect immunity (Buchmann 2014). The first line of defense of an insect against its environment is its cuticle, which acts as a physical barrier against most external factors (Dubovskiy et al. 2013). If this barrier is compromised, then pathogens are capable of exploiting a mosquito.
In addition, insects have also developed defense mechanisms to antagonize invading microorganisms internally, at the molecular level. These defense mechanisms represent the insect’s immune response.

Mosquitoes like all other insects have a specialized system of coordinating anti-pathogenic defenses. The innate immune system is one of the key mediators of interactions between mosquitoes and the pathogens that infect them (Clayton, Dong, and Dimopoulos 2014).

Upon infection, the innate immune system is capable of recognizing pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) that activate intracellular signaling cascades (Cirimotich et al. 2010). Once a signaling pathway has been activated, transcription factors translocate to the nucleus to regulate the production of immune gene mRNAs that code for PRRs, components of anti-pathogen effector mechanisms and immune activity regulators (Cirimotich et al. 2010).

There are three major signaling pathways that mediated anti-pathogen defenses in mosquitoes. These include the Toll pathway, the IMD pathway and the JAK-STAT pathway. The Toll and JAK-STAT pathways have been implicated in controlling dengue virus infection in the *Aedes* mosquito. However, many of the downstream effectors that mediate the antiviral activity are still largely unknown.
1.5 The JAK-STAT immune signaling pathway

The JAK-STAT signaling pathway shows some degree of conservation between mammals and insects, and plays different roles in their development. Studies on Drosophila immune pathways have guided the dissection of their orthologues in the mosquito immune system (Zdobnov et al. 2002). In Drosophila, the JAK-STAT pathway is activated when the protein unpaired (UPD) binds to Dome, a transmembrane receptor. The binding of UPD to Dome results in the transcription factor STAT inducing the expression of numerous genes including those involved in anti-pathogen defense (Arbouzova and Zeidler 2006). STAT transcription is negatively regulated by PIAS to avoid over activation of the pathway.

Our previous work suggested that the JAK-STAT pathway mediates part of its anti-dengue response via two molecules; dengue virus restriction factor 1 & 2 (DVRF1 and DVRF2) (Souza-Neto, Sim, & Dimopoulos, 2009). RNAi-mediated silencing of either gene resulted in a significant increase in dengue viral load in the mosquito’s midgut (Souza-Neto, Sim, & Dimopoulos, 2009). The mechanisms underlying JAK-STAT-mediated anti-pathogen defenses in the mosquito remain largely unexplored.

1.6 The microbiota

The environment shapes host-pathogen interactions. During dengue virus infection, the virus directly interacts with the mosquito's midgut environment. The midgut lumen consists of a rich array of microbes, such as bacteria and fungi that are known as the
mosquito midgut microbiota. Recent advances in our understanding of the mosquito microbiome have demonstrated that microbes play a major role in determining the outcome of pathogen infection in the mosquito. Research has revealed that some of the bacteria that reside in the midgut prime the immune defenses against invading pathogens. While the role of bacteria on infection outcomes have been studied to some extent, much less is known about the role of fungi in immunity and pathogen susceptibility.

Fungi are capable of producing a variety of proteins and secondary metabolites, which allows them to not only adapt to various environmental conditions, but also to act as opportunistic pathogens in insects and other organisms. During the initial stages of development, mosquitoes are exposed to fungi in the breeding water. As adults, mosquitoes can also be exposed to fungi via ingestion through nectar meals or by external physical contact with conidia (Tajedin et al. 2009; Lynch et al. 2012). So, the presence of fungi in the mosquito gut places them in proximity to invading pathogens. It is clear that fungi are naturally associated with mosquitoes, and further investigation into their potential for modulating mosquito-dengue interactions is warranted.
1.7 Specific Aims

Specific Aim 1: To characterize the role of a mosquito gut-associated fungus (belonging to the genus Penicillium) in priming and triggering mosquito immune responses, and influencing susceptibility to pathogens - covered in Chapter 2.

Specific Aim 2: To characterize the role of two dengue virus restriction factors (downstream putative effector molecules of the JAK-STAT immune pathway) in mosquito susceptibility to gram-positive and gram-negative bacterial infections - covered in Chapter 3.
Chapter 2: The mechanism through which *Penicillium decumbens*, an *Aedes aegypti* gut-associated fungus, enhances dengue virus infection

2.1 **Rational and Hypothesis:**

There has been extensive research aimed at characterizing mosquito gut-associated bacteria because of their ability to influence pathogen susceptibility of mosquitoes. Introduction of certain bacteria into mosquitoes through a blood meal may strongly alter the course of a pathogen infection of that mosquito (Bahia et al. 2014). For example, the *Anopheles gambiae* midgut bacterial microbiota significantly lower infection levels of the parasite, through the activation of basal immunity or the secretion of anti-parasitic molecules (Dong, Manfedini and Dimopoulos 2009). As such, exploitation of these mosquito-associated bacteria, that may impede the establishment of a pathogenic infection, could lead to the development of novel approaches for vector-borne disease control.

In contrast to the bacterial microbiota, much less is known about the mosquito gut-associated fungi, or mycobiome. In fact, many of the studies that have explored the use of fungi to control mosquito vector populations have only addressed entomopathogenic fungi (Fang et al. 2011; Bukhari, Takken, and Koenraadt 2011).

We were interested in understanding how different non-entomopathogenic fungi might influence the mosquito’s susceptibility to dengue virus infection. In order to assess this aspect in greater detail and to select fungi may mediate potent anti-dengue activity, we assayed the susceptibility of *Aedes aegypti* mosquitoes to dengue virus upon the
ingestion of several different cultivable fungi isolated from mosquitoes collected in the dengue-endemic region of Puerto Rico.

We next proceeded with the characterization of one of the isolated fungi, which was found to be of the *Talaromyces* species. We postulated that mosquitoes could be acquiring this fungus through feeding on contaminated nectar or water, and investigated its influence on dengue infection of the mosquito midgut.

### 2.2 Material and Methods

#### 2.2.1 Mosquito Rearing

Wild-type *Aedes aegypti*, Rockefeller strain mosquitoes were maintained on a 10% sucrose solution with 14-hr light, 10-hr dark cycles at 27°C and 80% humidity. At specific time points, mosquitoes were anesthetized on ice, and either whole mosquitoes (heads removed) or specific tissues were dissected and collected.

#### 2.2.2 Collection of Fungal Spores and Preparation of Filtrate

Fungal cultures were isolated and maintained at 4°C. From fungal stock, 5mL of Sabouraud Dextrose Broth (SDB) was then inoculated with and shaken at 27-30°C for up to 72 hours. Fungal suspension was then plated on 4% Sabouraud Glucose Agar (SIGMA) plates using sterile technique. After full confluency of agar plates, conidia were collected in 10 mls of 1x PBS containing 0.1% Tween 80 (Sigma). Glass wool was used
to filter out residual agar and mycelium from the solution. The solution was centrifuged at 2,000 rpm for 10 minutes. The pellet was then washed with 1x PBS and re-suspended in 1 ml of 1x PBS. The number of conidia in this solution was determined using a hemocytometer (Neubauer) under a 40x light microscope. For filtrate preparation, the spores were washed as described above, but then collected in a 10% sucrose solution. The suspension was then briefly vortexed and pelleted by centrifugation at 2,000 RPM for 10 minutes. After centrifugation, the supernatant was collected in a 1mL syringe and passed through a 0.2-micron filter (Millipore).

2.2.3 Conidia or Filtrate Introduced to Aedes by Sugar Meal

4-5 day old adult female *Ae. Aegypti* were starved for 4-6 hrs and then fed with the appropriate fungal treatment absorbed into cotton pads and placed in a location accessible to mosquitoes for 48 h. The cotton pads were subsequently removed and replaced with sterile 10% sucrose for the rest of the experiment.

2.2.4 Cell culture and Dengue virus challenge

The *Ae. albopictus* cell line (C6/36) was maintained in MEM (Gibco) supplemented with 10% FBS, 1% L-glutamine, 1% non-essential amino acids, and 1% penicillin/streptomycin. Baby hamster kidney cells (clone 15) (BHK-21) were maintained on DMEM (Gibco) supplemented with 10% FBS, 1% L284 glutamine, 1% penicillin/streptomycin, and 5 μg/mL plasmocin (Invivogen). C6/36 cells and BHK-21
cells were incubated in 5% CO$_2$ at 32°C and 37°C, respectively. *DENV2* strain New Guinea C (NGC) was propagated in C6/36 cells, and titers were determined using BHK-21 cells by plaque assay. Mosquito infections with dengue virus were carried out using a standard protocol (Das et al., 2007). The virus is first propagated in C6/36 cells for 6 days. A 1:1 virus suspension mixed with commercial human blood is prepared and supplemented with 10% human serum and 100 μM ATP. Mosquitoes were infected via an artificial membrane feeder at 37°C for 30 min or until fully engorged. Midguts were dissected and individually collected at 7 days post-infection.

2.2.5 *Dengue Virus enumeration in mosquitoes (Plaque Assay)*

BHK-21 cells were seeded on to 24-well plates overnight until 80% confluency. The next day, individual midguts that were dissected after dengue exposure, as previously described, were homogenized in DMEM with 0.5-mm glass beads using a Bullet Blender (Next Advance). The homogenates were then centrifuged at 14,000 rpm to obtain a virus-containing suspension. This suspension was then 10-fold serially diluted in 96-well plates. 100 uL of each dilution was then inoculated onto 80% confluent BHK-21 plates. The plates were rocked for 15 min at room temperature and then incubated for 45 min at 37°C and 5% CO$_2$. After the incubation, 1 mL of DMEM containing 2% FBS and 0.8% methylcellulose was added to each well. The plates were then incubated for 5 days at 37°C and 5% CO$_2$. Next, the plates were fixed and stained for 30 min in the incubator with a 1:1 methanol/acetone and 1% crystal violet mixture. After which the plates were washed with water and the plaque-forming units counted.
4-5 day old adult female mosquitoes were separated into two groups, one which was exposed and the other not exposed (control) to fungus filtrate for 48 hrs. Next both groups were given a non-infectious blood meal. Only fully engorged females from both groups were collected and maintained for on the normal sucrose feed for 6 days. Then their ovaries were dissected in 1x PBS, and the level of oogenesis was determined through microscopy. The least developed ovaries were given a score of 0, if some development (but not full development) was seen, these ovaries was scored as 1 and if full development was observed, these ovaries were scored as 2.

4-5 day old adult females were separated into two groups. The control group was maintained on a 10% sucrose solution, while the experimental group was maintained on fungus filtrate extracted in 10% sucrose overnight for two days. On the third day, both groups were feed with an artificial blood meal (40% PBS, 50% FBS, 1 mM ATP, and 2 mg of phenol red). This was done to avoid possible interference of blood heme with the assay. The next day, approximately 24 hours after blood feeding, mosquitos were dissected to extract their midguts in phosphate buffer (PBS, 10 mM Sodium Phosphate Buffer pH 7.2), and next placed in microcentrifuge tubes containing ice-cold reaction buffer (50 mM Tris- HCl pH 8.0, 10 mM CaCl2). Midguts were homogenized on ice with a pellet pestle, followed by centrifugation at 20,800 g at 4°C. The supernatant was flash
frozen in liquid nitrogen and stored at −80°C. The synthetic colorimetric substrate, Nα-benzoyl-D,L-arginine-p-nitroanilide hydrochloride (BApNA) (need to check order), was used to measure midgut extract trypsin activity based on the method of Erlanger et al. (Erlanger et al., 1961). Each reaction mixture contained 50 mM TRIS-HCl pH 8.0, 10 mM CaCl2, 1 mM BApNA, and 5μl of midgut extract. This reaction was incubated at 37°C for 5 mins and then the total trypsin activity of the midgut extracts was measured using a spectrophotometer; measured at A405nm.

2.2.8 In-vitro Trypsin Inhibition Assay

A reaction mixture was made using equal volumes of filtrate and trypsin. The mixture was incubated at (temperature of insectary) for at least 3 hours. Varying volumes of this mixture (10ul, 8ul, 6ul and 4ul) was added to 50 mM TRIS-HCl pH 8.0, 10 mM CaCl2, 1 mM BApNA and trypsin activity was measured using a spectrophotometer at 405nM.

2.2.9 Statistical Analysis

The Graphpad Prism 5 (Graphpad Prism®) software package was used to perform statistical analyses. The particular test used is indicated in the captions of each respective figure.
2.3 Different fungi can influence dengue virus titers in *Aedes* midguts

Here we investigated the effect of several non-entomopathogenic fungi on the modulation of *A. aegypti* susceptibility to dengue virus infection. For this we fed mosquitoes on a sucrose solution, enriched with whole fungus spores, for 2 days prior to a dengue virus infected blood feed. On day 7- post infection, mosquito midguts were dissected and dengue virus titers were measured. The results from this screen showed that the presence of two of the six tested fungi in the mosquito midgut, lead to a significant increase in dengue virus titers. One fungus decreased dengue virus titers in the midgut, while the remaining three fungi had no significant effect on dengue virus titers in the midgut.
**Figure 2.3.1:** Midgut dengue virus titers after feeding on different fungi isolated from mosquito midgut.

**Figure 2.3.1:** Dengue virus titers after feeding on different fungus isolated from a single mosquito midgut. Aedes mosquitoes were fungus-fed or fed for 48 h on a 10% sucrose solution containing $1 \times 10^9$ spores (A) No significant increase in dengue virus titers, upon exposure to fungi #7 and #8 (B) Dengue virus titers significantly decreased following feeding on fungi #20 but not fungi #22. (C) Dengue virus titers significantly increased following feeding on fungi #28 and #23. One biological replicate is shown in figure A. Two independent biological replicates were pooled for the assays in B and C. Statistical analysis of infection intensity was determined by Mann-Whitney analysis. Each dot represents a single midgut, and black horizontal bars represent the median (for (C) median = 0 for each).
2.4 Investigating the mechanism through which *P. decumbens* increases *Aedes* susceptibility to dengue virus infections

Given that *Aedes* exposure to different fungi led to differences in infection outcome, we decided to further investigate the mechanism through which one of the isolated gut-associated fungi mediated an increase in dengue titers.

Firstly, we investigated whether or not ingestion of fungal spores were necessary to mediate the effect. To do this we exposed adult female *Aedes* mosquitoes to (1) fungal spores in sucrose, (2) filtrate extracted from the fungal spores suspended in sucrose and (3) a sucrose solution as control. We found that filtrate exposure alone was sufficient to increase dengue infection of the mosquito midgut. This suggested that a molecule, or molecules, secreted by the fungus mediated the observed increase in dengue susceptibility.

To further investigate the mechanism through which the fungus exerted this activity, a microarray-based transcriptome analysis was conducted to investigate the influence of the fungi on the mosquito’s physiology. The assay revealed that a significant proportion of genes, that were functionally related to blood digestion, were down regulated upon fungal exposure.

*Talaromyces* species and other related fungi are known to produce a variety of proteins and secondary metabolites. Thus, we hypothesized that *P. decumbens* renders the mosquitoes more susceptible to DENV infection, through transcriptional inhibition of blood-digesting enzymes.

Based on these finding, we proceeded to investigate whether or not this effect could be measured at the protein/enzymatic level.
2.5  *Oogenesis as a measure for blood meal digestion*

Reproduction in adult female mosquitoes is tightly linked to their ability to transmit pathogens due to the fact that they require blood for egg development. Nutrients appear to be a key stimulus in alleviating the vitellogenic state of arrest in anautogenous mosquitoes. Specifically, nutrients derived from a blood meal triggers the activation of egg development. Since our previous experiment revealed no visual differences in the rate of blood meal digestion in the midgut, we decided to pursue another avenue as a measure for blood meal digestion success. We followed the same methodology as in the previous assay to create a control versus filtrate group, from which ovaries were dissected 6 days after blood meal ingestion. Our results showed normal egg development in most control mosquitoes, while there seemed to be little to no detectable egg development in filtrate-treated mosquitoes. We then decided to quantify the number of mosquitoes that portrayed the observed phenotypes by assigning an arbitrary score to different stages of development (0= no development (right image); 2=most developed (left image). There was a statistically significant difference in the level of ovary development between the mosquitoes in the control versus filtrate group, thereby suggesting that there was indeed a decrease in the mosquito’s ability to fully digest a blood meal after filtrate ingestion.
2.6 Midgut trypsin activity was significantly inhibited by *P. decumbens* filtrate

Midgut serine proteases play a central role in blood digestion by *Aedes aegypti* mosquitoes. They have also been implicated in affecting dengue virus infection of the midgut tissue. As previously mentioned, data from our transcriptome analysis showed the down-regulation of genes encoding trypsins and other important serine proteases. Here we performed an *in vivo* analysis of trypsin enzymatic activity using the same methodology to create a control versus filtrate group that were then individually given an
artificial blood meal. Midguts from these mosquitoes were dissected and homogenized, and added to a trypsin cleaving substrate. Midgut trypsin activity was significantly inhibited by *P. decumbens*. We also investigated whether or not the molecules secreted by the fungus could directly influence trypsin enzymatic activity *in vitro*. We performed an assay where we added various concentrations of a commercial trypsin to the same amount of a fungus filtrate, and then measured trypsin activity after 3 hours of incubation. This assay resulted in a significant decrease in *in vitro* activity of the commercial trypsin.

*Figure 2.6.1: Inhibition of *A. aegypti* midgut trypsin activity by Tsp_PR-secreted molecule*
2.7 Discussion

It is widely accepted that manipulating the mosquito vector to cure or prevent dengue infection will effectively break the cycle of transmission to humans. Current strategies to curb dengue transmission are all reliant on vector control, due to the unfortunate failure of the development of effective dengue vaccines and antiviral drugs the mosquito remains the most promising target for the reduction of vector borne dengue morbidity and mortality. The study of mosquito microbiota has resulted in new opportunities to exploit anti-pathogen defenses in order to interrupt dengue infection.

Our studies have revealed that there is yet another facet of the tripartite interaction between *Aedes*, dengue and *Aedes’s* microbiota that has not previously been studied. Exposure of *Aedes aegypti* mosquitoes to *P. decumbens* spores or secreted-molecules significantly increases dengue virus titers in *Aedes* midguts. In our efforts to elucidate the mechanism through which this effect occurs, we have shown that fungal interactions in the midgut can lead to changes in the mosquito, at both the enzymatic and the transcriptome level of trypsins.

An increase in the susceptibility of mosquitoes to dengue virus, as a result of fungal exposure, could translate into an enhanced viral transmission, although this possibility needs to be addressed experimentally. One strategy moving forward could be to correlate the presence between *P. decumbens* and dengue virus in field-caught mosquitoes in dengue-endemic areas.

However, given that mosquito egg production was severely affected by exposure to *P. decumbens* perhaps the amounts of filtrate used in this does not reflect a natural situation,
as it would impose a prohibitive fitness cost on the mosquito. As such, this dramatic
effect of enhanced infection may not occur in nature.

Also, our results have shown that different fungi have different influences on dengue
virus infection of the mosquito midgut, and our method of experimentations, that uses
single isolates, may not represent the interactions that take place in the mosquito
microbiota, that comprises several different species. However, a fungus that decreases
susceptibility to dengue infection could be developed into a novel strategy against dengue
transmission.
Chapter 3: Dengue virus restriction factor 1 is also implicated in antibacterial defense

3.1 Rational and Hypothesis:

*Aedes aegypti* mosquitoes modulate dengue virus infection primarily through their innate immune systems, which relies on the recognition of pathogen associated molecular patterns to activate various defense mechanisms. These defenses are primarily mediated via three signaling pathways, which include the JAK-STAT pathway, which has been implicated in controlling dengue virus infection in the *Aedes* mosquito. Downstream effectors that mediate the JAK-STAT pathway antiviral activity are still largely unknown. We previously showed that the JAK-STAT pathway mediates antiviral responses through two novel molecules, *DVRF1* and *DVRF2*. RNAi-mediated silencing of either gene resulted in a significant increase in viral load in the mosquito’s midgut. To begin to elucidate the mechanism through which the restriction factors function, we first begin to characterize the anti-pathogen specificity of *DVRF1* and *DVRF2*.

3.2 Materials and methods

3.2.1 Mosquito Rearing

Wild-type *Aedes aegypti*, Rockefeller strain mosquitoes were maintained on a 10% sucrose solution with 14-hr light, 10-hr dark cycles at 27°C and 80% humidity.
3.2.2 Primer Design

The Primer 3 Program (http://frodo.wi.mit.edu) was used to design DVRF1 and DVRF2 RNAi primers. PCR reactions were performed in triplicates, and melting curve analysis was used to analyze primer specificity.

3.2.3 RNAi Gene Silencing

Double-stranded RNA (dsRNA) was generated using the HiScribe T7 in vitro Transcription Kit (New England Biolabs) from PCR-amplified gene fragments. Gene silencing was performed as described in (Dong et al. 2006; Stéphanie Blandin et al. 2002). In summary, adult females were separated into a control or experimental group. These mosquitoes were then cold anesthetized, and each group was injected with control GFP dsRNA or experimental gene-specific dsRNA constructs at a concentration of 3µg/µL (207ng dsRNA per mosquito).

3.2.4 Bacterial Challenge

Three days post dsRNA-injection; mosquitoes were cold anesthetized and injected with 69 nL of either Gram-positive *Staphylococcus aureus* or Gram-negative *Escherichia coli*, at the optical density mentioned at the end of this section. Glycerol stocks of both species of bacteria were used to inoculate LB broth cultures, which were grown in a shaker overnight at 37° C for approximately 18 h. The cultures were centrifuged to a pellet that was then washed with 1x PBS three times. A biophotometer (Eppendorf) was used to measure optical density (OD600), and samples were diluted with 1x PBS to the appropriate absorbance prior to injection (OD600; *S. aureus* = 0.35, *E. coli* = 3.0)
3.2.5 Statistical Analysis

The Graphpad Prism 5 (Graphpad Prism®) software package was used to perform statistical analyses. The particular test used is indicated in the captions of each respective figure.

3.3 DVRF1 influences mosquito survival upon systemic infection with the gram-negative bacterium Escherichia coli.

Here we investigated the effect of Escherichia coli infection on the survival of Aedes aegypti mosquitoes after the silencing of DVRF1 and DVRF2 restriction factors. To do this we injected independent cohorts of adult female mosquitoes with synthesized DVRF1 and DVRF2 dsRNA and GFP dsRNA control. The mosquitoes were given 72 hours to recover and were then challenged with bacteria through thoracic injection. Mosquito survival was then monitored over several days. Systemic bacterial E. coli infections usually result in rapid mosquito mortality. RNAi mediated silencing of DVRF1 resulted in a significant reduction in the life span of A. aegypti over the time period of a week, while silencing of DVRF2 had no significant effect on the life span of the mosquitoes tested.
3.4  **DVRF1 and DVRF2 have significant effects on mosquito survival after systemic infection with the gram-positive bacterium *Staphylococcus aureus***

Here we investigated the effect of systemic *Staphylococcus aureus* infections on the survival of *Aedes aegypti* mosquitoes after the silencing of *DVRF1* and *DVRF2*. The same methodology previously described was utilized. Systemic *S. aureus* infections also resulted in mortality over a 10-day time period. RNAi mediated silencing of both *DVRF1*
and DVRF2 prior to bacterial challenge resulted in a significant reduction in the life span of adult female *A. aegypti* mosquitoes.

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**Fig 3.4.1: Survival assay - *S. Aureus*.** Female mosquitoes were cold anesthetized and injected with DVRF1 and DVRF2 dsRNA. The mosquitoes were given a few days to recover, after which, they were challenged by injection with *S. Aureus*, and survivability monitored daily for a total of 10 days in three independent experiments (N=50, P = 0.0118, P = 0.0037).
3.5 Discussion

The *Aedes* mosquito’s anti-dengue defense system is actively engaged in limiting viral infection of the midgut epithelium. While these immune responses have been shown to be regulated to some extent by the JAK-STAT pathway, here we show yet another uncharacterized function of the downstream dengue effector molecules that also participate in limiting bacterial infection.

For the first time we show that the pathogen specificity of DVRF1 and DVRF2 is not limited to dengue. DVRF1 influences mosquito survival upon systemic infection with *E. coli* while both DVRF1 and DVRF2 influences mosquito survival upon systemic infection with *S. Aureus*.

The effect of the silencing of DVRF1 and DVRF2 genes leading to a decrease in survivability against systemic bacterial infection suggests that these genes may not be limited to anti-viral defenses only.

The future direction of this project would be to design gain of function experiments by creating a transgenic *A. aegypti* mosquito line that ectopically overexpresses DVRF1 or DVRF2. Susceptibility to viral and bacterial infections will be assessed again using our previously described viral and bacterial challenge methods. The results of these experiments will corroborate our conclusions as to whether or not DVRF1 and DVRF2 are broad-spectrum defense mediators of *A. aegypti* mosquitoes, or if their functions are specifically targeted towards the mosquito’s anti-dengue defense.
Supplementary Figures and Tables

Table S1: Primers used to develop dsRNA

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<th>Gene Target</th>
<th>Primer Name</th>
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<td>DVRF1-Forward</td>
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S1: DVRF1 & DVRF2 dsRNA gel

Figure S1: DVRF1 & DVRF2 dsRNA gel. PCR product for dsDVRF1 and dsDVRF2
References


Buchmann, K. (2014). Evolution of innate immunity: clues from invertebrates via fish to
mammals. *Frontiers in immunology, 5.*


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http://www.who.int/mediacentre/factsheets/fs117/en/

Education

**ScM. Student Molecular Microbiology and Immunology, August 2017, Johns Hopkins University, Baltimore, MD**

**B.S., Biochemistry, May 2014, Syracuse University, Syracuse, NY**

*Distinction in Biochemistry, Magna Cum Laude*

Work Experience

Student Research Assistant, Johns Hopkins Bloomberg School of Public Health - *July 2015- January 2016*

- Conducted systematic reviews on interventions for global child injuries prevention

Founding member and US coordinator, Project Pikin - *April 2015 – December 2015*

- Organized fundraising events, clothing and book drives for children who had been orphaned or lost a parent or guardian during the Ebola outbreak in Sierra Leone

Laboratory Intern, Central Public Health Reference Laboratory for HIV and Other Infectious Diseases, Freetown, Sierra Leone - *May – July 2011*

- Assisted in testing samples for standard HIV Diagnosis via PCR testing and ELISA.
- Attended and participated in bi-weekly meetings on infectious diseases update with the Ministry of Health
- Provided bi-monthly laboratory support to the Mercy Ship volunteers

Research Experience

Graduate Student, *Johns Hopkins Bloomberg School of Public Health, Baltimore, MD*

*August 2014- August 2017*

- Quantified native and recombinant Histidine-rich Proteins used as a sensor of
- Characterizing the JAK-STAT pathway regulated dengue virus restriction factors to elucidate mosquito antiviral defenses.
- Studying the influence of mosquito-associated fungi on the susceptibility to the dengue virus.
- Design and utilize a HBV pseudoparticle system to study neutralizing antibody response produced by acute and chronically infected individuals.

Undergraduate Student, *Syracuse University, Syracuse, NY*

Studied Interneuron specification in the zebrafish spinal cord - *March 2012-May 2014*
• Identified regulatory genes expressed by interneurons with ipsilateral axon morphologies using *in situ* hybridization, antibody staining and microarray analyses data.

• Characterized the expression patterns of spinal cord *Ladybird Homeobox* genes in Zebrafish

**Volunteer**

Volunteer, Johns Hopkins Institutes of Behavior Resources-REACH health literacy program- *February 2016- May 2016*

• Assisted a SOURCE scholar to design and administer health literacy classes to patients with a history of substance use

Volunteer, Syracuse City High Schools- *2012-2014*

• Supported the teaching of underrepresented minority high-school students scientific research methods

• Administered college entry Q&A sessions, both on campus and at Syracuse city high schools

• Served as a mentor to excited/motivate Syracuse city high school students to pursue careers in science

Ambassador and Mentor, Syracuse -Danforth and Blodgett Schools, and at the Hillside Work-Scholarship Connection - *February – April 2011*

• Performed science experiments with children in 6th-8th grade in the Syracuse City School District’s after-school science clubs

• Strived to inspire the students to follow careers in math, science and technology through high school and beyond

**Funding and Awards**

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<td>Syracuse University PRIDE Circle of Excellence</td>
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<td>The Otis and Calista Causey Fellowship for Immunology</td>
<td>2015/2016 and 2016/2017</td>
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**Publications**

Technical Skills

*Software:* MatLab; HyperChem; STATA

*Languages:* Fluent in English & Krio, conversational in French