Bacteria-delivered RNA interference strategies to silence genes related to vector competence of *Aedes aegypti*

by

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Abstract

Dengue infection is a devastating mosquito-borne disease, and the principal vector is the mosquito, *Aedes aegypti*. Current vector control strategies are not working, hence the need for alternative strategies. Cathepsin B is a mosquito protein that dengue viruses require to establish and replicate within mosquitoes; knocking down cathepsin B using RNAi changes the phenotype from dengue susceptible to dengue refractory in *Ae. aegypti*. We engineered bacteria to express dsRNA against cathepsin B to develop an orally delivered RNAi system. Our data suggest inconsistencies in the alteration of gene expression that may be a result of the modified bacteria being digested, or a lower than required quantity of the RNAi constructs being expressed. Without a consistent knockdown, it is unlikely that we will be able to reduce vector competence predictably.

Keywords: *Aedes aegypti*; dengue; bacteria-delivered dsRNA; cathepsin B; fitness test; antibiotic treatment

Dedication

This thesis is for all the dengue victims around the world.

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Chapter 1.

Introduction

1.1. Dengue infection

1.1.1. General

Dengue fever is a mosquito-vectored disease that is found mainly in the tropics and subtropics [1]. Dengue viruses (DENV) can be transmitted through sylvatic and urban cycles, between mosquito vectors and vertebrate hosts including humans and lower primates [2]. The mosquito vectors belong to the genus *Aedes,* primarily *Aedes aegypti* and *Aedes albopictus*. Clinical manifestations of dengue infection range from silent infections with no symptoms to mild flu-like syndrome, dengue fever, and severe dengue diseases that are life-threatening, such as dengue haemorrhagic fever and dengue shock syndrome [3].

Dengue virus is a member of the family *Flaviviridae* which comprises enveloped viruses with positive single-stranded RNA genomes [4]. There are 4 genetically related, but antigenically distinct, serotypes of dengue virus: DENV-1, DENV-2, DENV-3 and DENV-4 [5]. Recovery from infection by one serotype provides lifelong immunity against that particular serotype. Subsequent infections by other serotypes, however, may lead to severe dengue diseases due to antibody-dependent enhancement [5]. Antibody-dependent enhancement occurs when pre-existing antibodies from a primary dengue infection bind but do not neutralize dengue virus of a different serotype during a subsequent infection [6]. Instead, the antibody-virus complexes enhance the infection of circulating monocytes by attaching to their Fcγ receptors. As a result, there is an increase in the overall replication of viruses, proliferation of T cells and production of pro-inflammatory cytokines, causing severe Dengue disease [6]. Unfortunately, there are no effective vaccines that protect well against all 4 serotypes of DENV and no drugs to eliminate DENV [7,8]. Control efforts have continued to rely on vector control strategies to reduce vector populations and reduce the transmission of DENV.

1.1.2. Global distribution

Approximately 390 million people are infected with dengue annually, which results in more than 25,000 deaths [9,10]. Over half of the global population in 128 countries is at risk of dengue infection (Figure 1.1) [11]. Despite intensive efforts, the burden of dengue infection has increased 30-fold over the past 50 years [1]. The regions of highest risk for dengue are in Asia and the Americas, contributing 70% and 14% of the global burden respectively. The remaining 16% are in Africa, but this could be underestimated due to poor documentation of data in Africa [1].

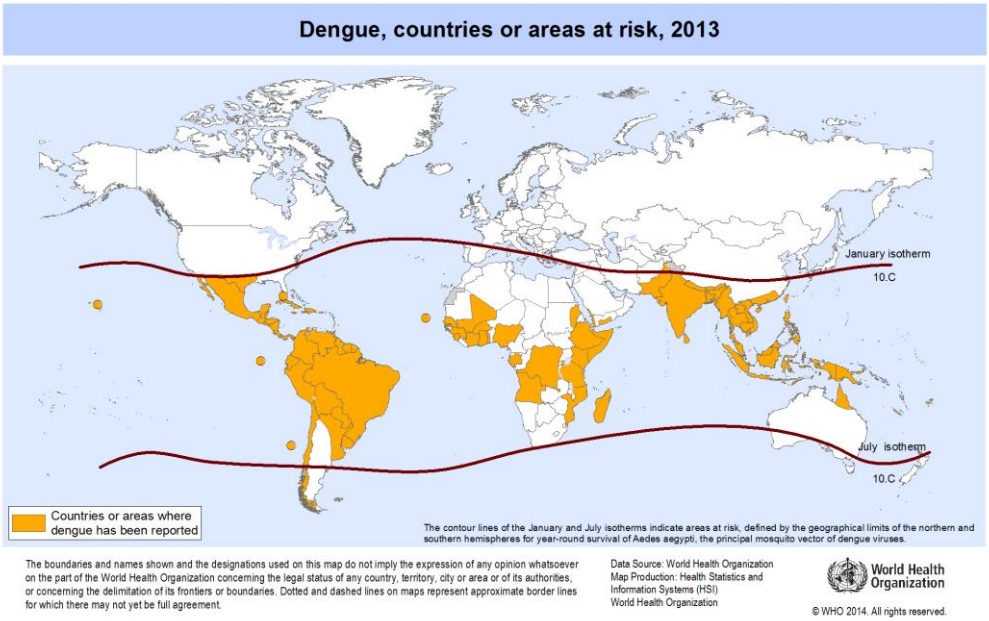

Figure 1.1 Countries or areas at risk of DENV infection. This figure is obtained from Health Statistics and Information System, World Health Organization [12].

The distribution of dengue corresponds directly to the distribution of mosquito vectors [13]. While *Ae. aegypti* is a tropical and subtropical mosquito, it has spread into new geographical areas largely due to human activities such as international shipping [14,15]. The eggs of *Aedes* sp. can survive months before hatching, which allows them to be transported long distances. The global shipping of rubber tires resulted in the establishment of an invasive species, *Aedes albopictus*, in the southern USA [16]. As models predict increases in temperature and rainfall due to climate change, conditions will become perfect for range expansion of many vector species [11].

1.1.3. Transmission

Dengue virus can be transmitted through two different life cycles: sylvatic and urban cycles. The sylvatic cycle occurs between mosquitoes and non-human primates, whereas the urban cycles occur between mosquitoes and humans [2].

A mosquito acquires DENV by ingesting a bloodmeal from an infected host. The bloodmeal enters the midgut lumen, where viruses infect midgut epithelial cells [17]. Virus replication occurs in the midgut epithelial cells 24 hours to 72 hours after ingestion after which they spread to secondary tissues such as muscles, nerves, fat body, tracheae, ovaries, and haemocytes [18]. Approximately two weeks after being ingested by the vector, DENV moves to the salivary glands [19]. Once the salivary glands are infected, the virus replicates and can be transmitted to a new host during feeding.

1.1.4. Dengue Virus

Genome and structure

The DENV genome is a single strand of positive-sense RNA that encodes 10 genes (Figure 1.2). Positive-sense RNA can be translated directly as a single, long polypeptide, which then is processed by cellular and viral proteases into 10 proteins [20]. The 10 proteins comprise 3 structural proteins (capsid (C), envelope (E), and membrane (M) proteins) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Non-structural proteins are involved in viral replication and assembly.

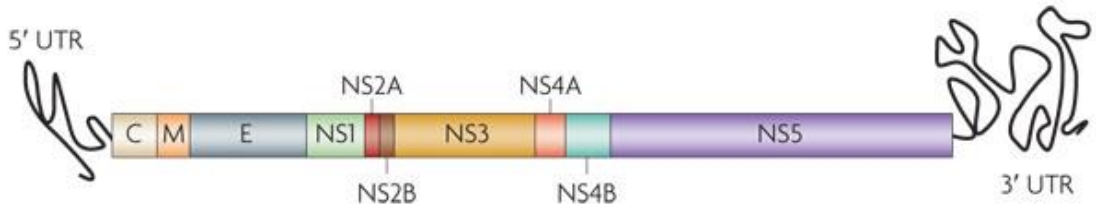

Figure 1.2 Dengue virus genome. This figure is obtained from the article "Dengue: a continuing global threat" [20].

DENV has a diameter of approximately 50 nm and a roughly spherical shape. The spherical nucleocapsid core comprises the viral genome that is encapsulated by multiple copies of C proteins [21–23]. The nucleocapsid is surrounded by the viral envelope,

which is a host cell lipid bilayer embedded with 180 copies of M and E proteins [24,25]. Both M and E proteins control the entry of DENV into the host cell.

Replication and infectious cycle

The replication of DENV is shown in Figure 1.3. To enter a cell, DENV attaches to multiple receptors such as heat-shock protein 70 (Hsp70), R80, R67, and a 45-kDa glycoprotein on mosquito cells [26–28]. Once the virus attaches to membrane receptors, it migrates on the cell surface by rolling over different receptors or by migrating as a virus-receptor complex, until it reaches a clathrin-coated pit [29]. Then, the virus is engulfed by the cell via clathrin-mediated endocytosis [30].

Within the endosome, the nucleocapsid is released into the cell cytosol, where nucleocapsid opens to release the viral genome [29,31,32]. DENV hijacks the host cell's machinery to replicate and translate its genome [32]. Immature viruses become mature in the ER as they travel through the secretory pathway. At first, slightly acidic pH of the trans-Golgi network (TGN) triggers E proteins to dissociate from prM proteins, forming E homodimers that lie flat on the virion surface, with prM proteins capping the fusion peptide of E proteins [33–35]. This conformational change enables cellular endoprotease furin to cleave pr peptides out of prM. Virions are considered mature after dissociation of pr peptides and are capable of infecting other cells once they exit from the cell.

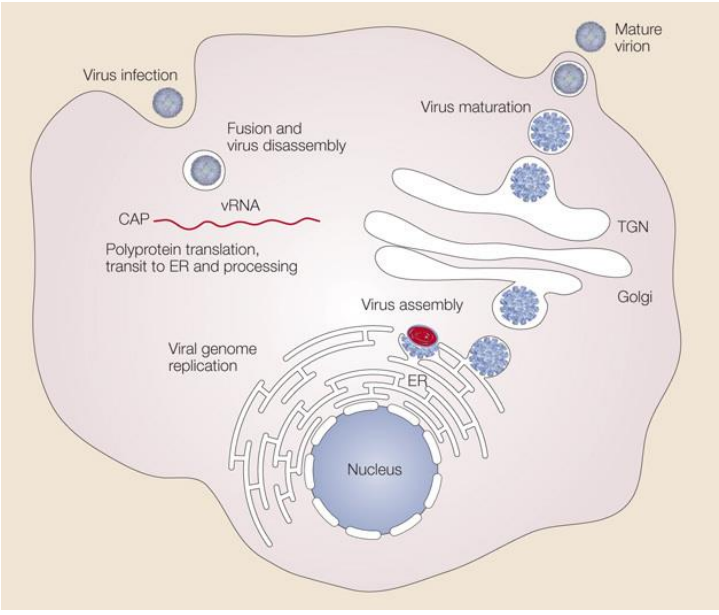

Figure 1.3 Infectious cycle of dengue virus. This figure is obtained from the article "A structural perspective of the flavivirus life cycle" [36].

1.1.5. *Aedes aegypti*

The distribution of dengue infection is closely linked to the global distribution of *Ae. aegypti*, the primary vector of DENV. This species also is the principle vector of other arthropod-borne viruses (arboviruses) including yellow fever virus, chikungunya virus and Zika virus to humans [37].

Life History

Mosquitoes are holometabolous insects; they undergo four stages of development: eggs, larvae, pupae, and adults (Figure 1.4). Eggs, larvae, and pupae are aquatic, whereas adult mosquitoes are terrestrial. Eggs can survive desiccation for several months and then hatch immediately after submersion in water, producing first stage larvae that consume bacteria and plankton [1,38]. Larvae moult through 4 larval instars as they accumulate sufficient resources, and fourth instar larvae moult into pupae, a non-feeding aquatic stage [39]. During the transition from pupa to adult, all the larval structures are modified or resorbed and the adult structures, mouthparts, wings, halteres, and legs, and digestive tract are synthesized [39]. Finally, an adult emerges from the pupa and disperses from the larval habitat. Both females and males feed on nectar and plant sap and are capable of hydrolyzing sucrose, but only females are hematophagous [40].

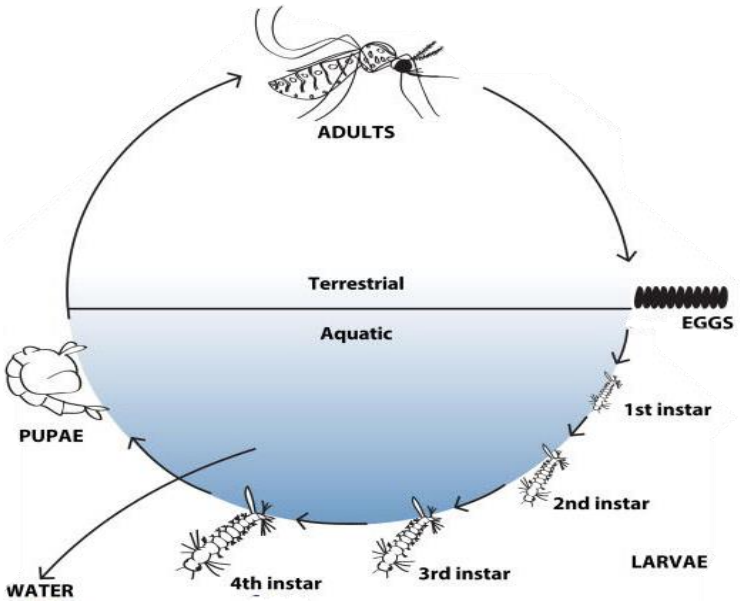

Figure 1.4 Life cycle of *Ae. aegypti*. This figure is modified from the article "Mosquitoes rely on their gut microbiota for development" [41].

Blood-feeding and digestion

Aedes aegypti is found commonly in urban environments in close contact with humans; in fact, humans are their preferred hosts, which leads to high transmission rates of pathogens such as DENV [42]. Blood-feeding is also a dangerous activity as many hosts try to kill the mosquitoes, but blood-feeding is an essential activity as the blood provides the proteins required to produce offspring [40].

After a bloodmeal is taken, the mosquito midgut is hugely distended. Within an hour, water is excreted via the Malpighian tubules, making the bloodmeal more concentrated [43]. The bloodmeal is surrounded by a peritrophic matrix, secreted by the midgut, that helps isolate proteolytic enzymes from inhibitors that are present within the bloodmeal [43]. Digestive enzymes such as trypsin and aminopeptidase are secreted into the ectoperitrophic fluid, between midgut epithelium and the peritrophic matrix [43]. Digestion proceeds inwards from the periphery of the bloodmeal, while digested food is being absorbed. Essential amino acids are absorbed by midgut epithelial cells, transported and taken up in the fat body [44]. Lipids and sugars are absorbed in the midgut and transported to the hemolymph [45].

Oogenesis and oviposition

Blood-feeding by the newly emerged *Ae. aegypti* female induces the release of two neurohormones from the brain: insulin-like peptides (ILPs) and ovary ecdysteroidogenic hormone (OEH). These hormones stimulate the ovaries to produce ecdysone, which triggers the fat body to produce yolk proteins [46–49]. Yolk proteins then can be packaged into primary oocytes to form mature eggs. Meanwhile, the fat body also secretes cathepsin B, a latent proenzyme that is accumulated and stored in yolk bodies. Cathepsin B degrades vitellogenin, a yolk protein precursor, and vitellin, a major yolk protein in eggs [50].

Approximately 3-4 days after blood-feeding, adult females choose a suitable oviposition site to lay their eggs [51]. *Aedes aegypti* prefers to oviposit in water that contains healthy conspecific larvae and coliform bacteria [52–54]. The data suggest that females avoid ovipositing in poor quality habitats containing stressed larvae that are crowded, have little food, or contain predators and parasites [52,55,56]. *Aedes aegypti* prefers to oviposit in small containers of transient clean water, flower pots, discarded

cartons, etc. in contrast to other species such as *Anopheles gambiae* that lays eggs in permanent water sites such as ponds [13,57].

1.2. Dengue control

1.2.1. Treatment and vaccine

There are no drugs available to eliminate DENV. However, early detection and proper medical care can decrease fatality rates below 1%, mostly by maintaining the patient's body fluid volume [8]. An effective dengue vaccine must provide strong protection against all 4 serotypes at once to reduce the risk of developing severe dengue disease via antibody-dependent enhancement. In 2015, Dengvaxia vaccine became the first licensed vaccine in the world for dengue prevention [58]. This tetravalent chimeric vaccine was made by substituting the prM and E genes from each of the four dengue serotypes into the backbone of the yellow fever virus 17D vaccine strain [59,60].

Unfortunately, Dengvaxia vaccine has limited efficacy. Although this vaccine is highly effective against DENV-3 and DENV-4 serotypes, it provides modest protection against DENV-1 and less protection against DENV-2 [61,62]. In addition, the efficacy of Dengvaxia depends strongly on the age of vaccinated recipients and local transmission intensity [63]. Reports have shown that vaccinated children who were younger than 9 years old had increased rates of hospitalization for severe dengue symptoms [64]. Vaccination in locations with low DENV transmission intensity also had increased incidence of severe dengue diseases [63]. In contrast, vaccination was effective for both seropositive and seronegative recipients in high-transmission settings. In moderate transmission settings, seropositive recipients were protected by the vaccine, but seronegative recipients had an increased risk of hospitalization with severe dengue [63].

1.2.2. Vector Control

Traditional strategies

Traditional vector control strategies include the application of chemical insecticides and larvicides or the elimination of egg-laying habitats [8]. Although these strategies are used widely, they need improvements. Due to continuous and excessive use of insecticides, *Ae. aegypti* has evolved resistance to many commonly used

insecticides [65–67]. Furthermore, insecticide applications may affect non-target organisms [68]. Since mosquitoes can breed in small dispersed bodies of water such as buckets and vases, there are large numbers of potential breeding sites in urban areas making it impossible to eliminate egg-laying habitats.

Alternative strategies

Due to the ineffectiveness of traditional strategies, recent research focus has emphasized alternative strategies such as biological control (biocontrol) and genetic manipulation. The goals of these approaches are to reduce the populations of vectors or to render these populations incapable of harbouring and transmitting the pathogens.

Biocontrol

Biocontrol involves the introduction of natural enemies to reduce or eliminate pest populations. This approach is often more environmentally friendly and sustainable than the application of pesticides [69]. For example, copepods that prey on mosquito larvae are very effective biocontrol agents. The application of copepods in Vietnam successfully eradicated *Ae. aegypti* from large surrounding areas within two years [70]. However, this biocontrol strategy has limitations because larval habitats are not suitable for copepods. For the same reason, larvivorous organisms such as amphibians and fish are not suitable to be used as biocontrol agents against *Ae. aegypti* in very transient urban areas.

Other potential biocontrol agents are mosquitoes from the genus *Toxorhynchites*, because their larvae prey on larvae of other mosquito species [69]. *Toxorhynchites* adults feed on honeydew, fruit, and nectar instead of blood. Thus, they are mosquito eaters that are harmless to humans [69]. Nevertheless, further investigation on the potential environmental threat of introducing these mosquitoes is required.

Other biocontrol strategies include the use of microorganisms as pesticides. For example, a fungus, *Metarhizium anisopliae,* can be cultivated to kill *Ae. aegypti* larvae and adults [71]. *Bacillus thuringiensis serovar israelensis* (Bti), a bacterium that can produce toxins encoded by cry and cyt genes on a plasmid, can kill mosquito larvae by degrading their midgut membranes [72,73]. Bti and other similar biological insecticides have been used to reduce *Ae. aegypti* populations, but it is difficult to deliver Bti to transient

larval habitats and now problems have arisen due to the development of resistance to Bti [69].

Paratransgenesis

Rather than eliminate populations, recent research has focused on developing techniques to reduce the vector competence of mosquitoes; the innate ability of a vector to acquire, maintain and transmit pathogens [74]. One approach is to modify genetically the normal mosquito microbiome via paratransgenesis [75,76]. Symbiotic viruses such as Sindbis viruses have been engineered to express transcripts from DENV that can prevent the replication of DENV in mosquitoes [77]. The alpha-proteobacterium *Asaia*, which colonizes in the gut, salivary gland, and reproductive organ of both female and male *Ae. aegypti* can be potentially used for paratransgenesis [78]. *Asaia* is found in all the developmental stages of *Ae. aegypti* with very high prevalence [78]. A paratransgenesis approach has been used to generate modified *Rhodococus* sp., an obligate bacterial symbiont of *Rhodnius prolixus*, to express antimicrobial peptides that kill *Trypanosoma cruzi*, a parasite that kills ~20,000 people each year [79,80]. Similar approaches have been proposed or are being developed to control mosquitoes [81], tsetse flies [82], and the parasites they transmit.

Another approach has transfected *Ae. aegypti* with an endosymbiotic bacterium, *Wolbachia pipientis,* that can shorten the lifespans of mosquitoes and reduce vector competence by interfering with DENV replication and dissemination [83–85]. This bacterium also can reduce viral loads of other viruses such as chikungunya and Zika in *Ae. aegypti* [83–85]. Field studies are underway in South East Asia, India, and South America using "Wolbachia" mosquitoes to determine if the transmission can be reduced significantly.

Genetic manipulation

Mosquitoes also have been modified genetically to incorporate desired molecules or lethal genes. Commercially produced insects have engineered to contain a dominant lethal gene (Release of Insects Carrying a Dominant Lethal [RIDL]) that kills the mosquitoes in the larval stage. [86–88]. This approach has been developed and marketed commercially by Oxitec and is being used to reduce *Ae. aegypti* populations in areas with limited immigration potential such as islands [89].

Other gene manipulation strategies utilize single-chain antibodies, innate immunity peptides, artificial peptides, mutagenesis, altered cellular signaling, and RNA interference [85,90,91]. Mutagenesis in *Ae. aegypti* has been established to generate refractory mosquitoes. Commonly used mutagenesis methods include transposon-mediated transgenesis, loss-of-function gene editing with zinc-finger nucleases (ZFNs), TAL effector nucleases (TALENS), and homing endonuclease genes (HEGs) [92–99]. ZFNs and TALENs are modular DNA-binding proteins designed to bind to a nonspecific FokI DNA nuclease, whereas HEGs are endonucleases that are modified to target DNA sequences [100,101]. These methods are target specific and depend on protein-DNA-binding interactions. In late 2012, a more efficient and flexible genome editing system was developed: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) genes. The CRISPR-Cas9 system was discovered in wide varieties of adaptive immune systems in bacteria and archaea [102]. Bacterial type II CRISPR-Cas9 system can be used to generate site-specific mutations by relying on RNA-DNA base pairing [102].

RNA interference

RNA interference (RNAi) is a naturally occurring phenomenon discovered in the nematode *Caenorhabditis elegans* [103,104]. In brief, RNAi employs double-stranded RNA (dsRNA) to degrade endogenous mRNA, leading to a reduced expression of the corresponding gene (Figure 1.5).

In insects, RNAi is an intracellular antiviral immune response used to eliminate foreign RNA, particularly double-stranded virus RNA [106]. Virus dsRNA is recognized and cleaved by an RNase III family dsRNA endonuclease called Dicer-2 to generate 21-25 nt long short interfering RNA duplexes (siRNAs) [107]. These siRNAs are then incorporated into the RNA induced silencing complex (RISC) by the Dicer-2/R2D2 complex [108]. Following the removal of the passenger strand via the RNase activity by Argonaute-2 and the endoribonuclease C3PO, RISC uses the remaining siRNA strand as a guide to bind to the complementary viral mRNA and degrades it via Argonaute-2-mediated cleavage, preventing translation [106,109,110]. Due to its simplicity, specificity, and effectiveness, RNAi is used commonly for gene functional determination and gene silencing, in medicine for cancer and viral disease treatment, and as a promising tool for pest control in agriculture [111–113]. For insect pests, RNAi is a great alternative to pesticide

application because RNAi can be target specific, environmentally friendly, and from the perspective of this study, can be used to inhibit the replication of viruses in mosquito vectors [18,114].

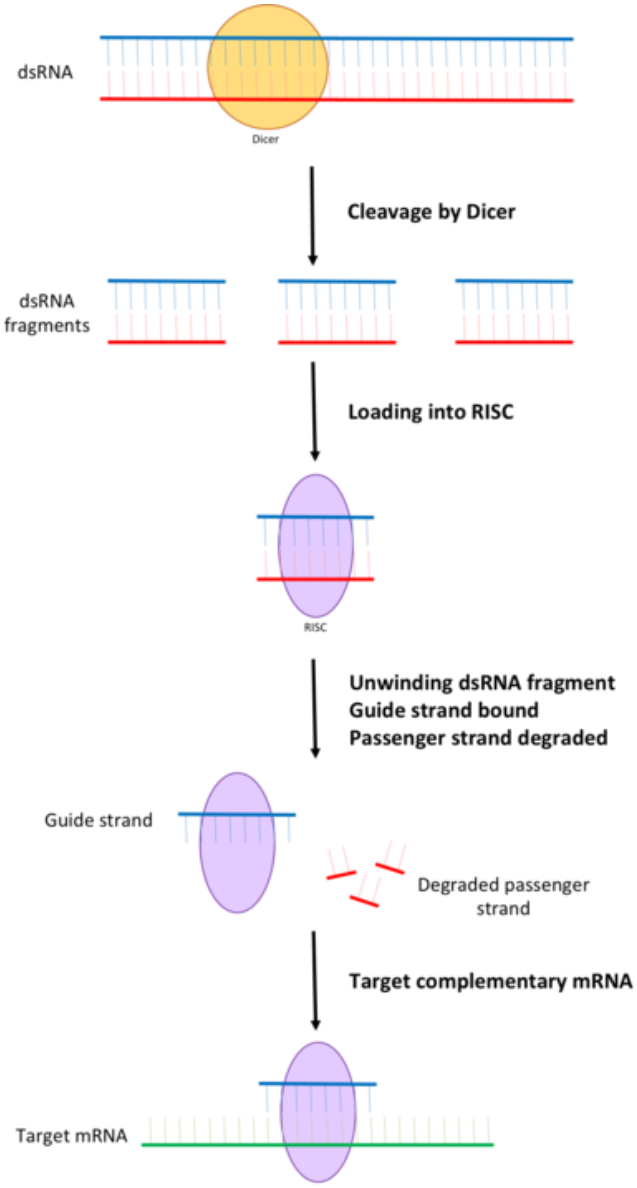

Figure 1.5 The mechanism of RNA interference. This image is modified from Wikimedia Commons [105].

In most laboratory studies, dsRNA is injected into insects and the knockdown (Kd) effect is quantified. These techniques have been used extensively to study gene expression and the specific roles of selected mosquito genes [76,115–117]. However, microinjection has many limitations, including the need for skilled personnel and special

equipment. Another approach is to use bacteria to express dsRNA in the target insect species.

Escherichia coli-mediated delivery of dsRNA was first used in *C. elegans* in 1998 [118]. In this approach, the target knockdown genes were flanked by bidirectional bacteriophage T7 promoters, inserted into cloning vectors, and transformed into bacteria with an inducible T7 polymerase. Once induced, bacteria produce and secrete the dsRNA. Bacteria-delivered dsRNA can be used for specific applications and specific insect targets. In *Rhodnius prolixus*, an insect vector of the human parasite *Trypanosoma cruzi*, bacteria were engineered to express dsRNA that targeted the *Rhodnius* haem-binding protein (RHBP) and catalase genes. The knockdown of these genes reduced vector longevity and viability of eggs produced by the females [119]. This is the approach we copied for our studies described below.

Refractory mosquitoes

Although *Ae. aegypti* is the principal vector of DENV, not all mosquitoes transmit DENV. In Cali, Colombia, approximately 30% of all female *Ae. aegypti* collected in the field are refractory to DENV: they kill the virus through mechanisms described as midgut infection barriers (MIB) or midgut escape barriers (MEB). In these insects, the virus cannot enter and replicate within midgut cells (MIB), or if it does, it cannot escape from these cells (MEB) [18,120,121]. Differential display, microarrays, and RNA sequencing (RNA seq) were used to identify the mechanisms that contribute to the refractory phenotype [18,116,122,123]. Several overexpressed genes were identified in the susceptible mosquitoes that might favour viral replication and dissemination in the mosquitoes. Some of these genes were knocked down by the injection of dsRNA. RNAi targeted knockdown of these genes changed the phenotype: the susceptible strain (Cali-S) became refractory and was indistinguishable from the refractory strain (Cali-MIB) [18]. One of these genes was cathepsin B.

Cathepsin B is a lysosomal cysteine protease that is involved in, among many other functions, the apoptosis of immune cells [124]. ROS-dependent release of cathepsin B into the cytosol induces the cleavage of pro-apoptotic a Bcl-2 family member (Bid), leading to mitochondrial damage, caspase activation and finally apoptosis [125]. In addition, cathepsin B is required for the proteolytic cleavage of Toll-like receptor (TLR) 7 and TLR 9, which are essential for recruitment of the signaling adaptor MyD88 [126,127].

Cathepsin B also is involved in the posttranslational processing and production of TNF-alpha, in response to bacterial cell wall component lipopolysaccharide (LPS) [128]. The role of apoptosis was characterized in the Cali-S and Cali-MIB strains[122] leading to the idea that using bacteria to express dsRNA to target the cathepsin B gene might provide a novel way to deliver dsRNA and reduce the susceptibility of *Ae. aegypti* to DENV, and potentially to other similar arboviruses [122].

1.3. Research objectives

The main objectives of this research are to:

1) generate bacteria that express dsRNA that will knock down the expression of cathepsin B in midguts of *Ae. aegypti* to a level that will affect the phenotype

2) determine whether ingestion of bacteria that express dsRNA of cathepsin B negatively affects the fitness of *Ae. aegypti* including fecundity, egg viability, and longevity

3) determine if we can increase the proportion of dsRNA expressing bacteria in the midguts of *Ae. aegypti* by pre-treating them with antibiotics

Chapter 2.

Using bacteria-delivered dsRNA to reduce the expression of cathepsin B in *Ae. aegypti*

2.1. Introduction

Dengue fever, dengue haemorrhagic fever and dengue shock syndrome, caused by infection with dengue viruses (DENV) have become the most common mosquito-borne viral diseases [9,10]. *Aedes aegypti* is the main vector of DENV [3], and efforts to reduce disease have been based on reducing *Ae. aegypti* populations. Unfortunately, current vector control strategies, based on insecticide application and elimination of egg-laying habitats, are not effective, hence the need for alternative vector control strategies. These include biocontrol, paratransgenesis, genetic manipulation and RNA interference. The main goals are to reduce vector populations or vector competence, the innate ability of a vector to acquire, maintain and transmit pathogens [74].

Not all *Ae. aegypti* females, however, transmit DENV. Our previous studies demonstrated that ~ 30% of all wild *Ae. aegypti* females collected in the field around Cali, Colombia were refractory to all four serotypes of DENV; they killed the virus through a midgut infection barrier (Cali-MIB) or a midgut escape barrier (Cali-MEB) [120,121]. Members of the Lowenberger and Ocampo labs used whole-genome microarrays [17] and RNA seq approaches to compare gene expression in susceptible (Cali-S) and refractory (Cali-MIB) females that fed on either sugar, blood, or blood infected with DENV-2 [18,123]. They identified a series of genes that were differentially expressed in the two strains, and which might affect the vector competence of *Ae. aegypti* [116,122,129]. They used these genes in functional RNAi studies: they injected dsRNA into female *Ae. aegypti* to knock down the expression of selected genes in the Cali-S strain and this altered the phenotype: susceptible mosquitoes became refractory [18]. One of these genes was cathepsin B that is involved in the degradation of host hemoglobin in blood-sucking insects [130]. Cathepsin B also is secreted as a proenzyme by the fat bodies of females during vitellogenesis and accumulates in developing oocytes. Cathepsin B is involved in innate immune responses; it is required for the proteolytic cleavage of Toll-like receptor (TLR) 7 and TLR 9, which are essential for recruitment of the signalling adaptor MyD88

[126,127]. Cathepsin B is involved in the posttranslational processing and production of TNF-alpha, in response to bacterial cell wall component lipopolysaccharide (LPS), and also is involved in the apoptosis of immune cells [124,128]. ROS-dependent release of cathepsin B into the cytosol induces the cleavage of pro-apoptotic a Bcl-2 family member (Bid), leading to mitochondrial damage, subsequent caspase activation and finally apoptosis [125].

The knockdown studies described above, however, required an injection of dsRNA into the body cavity of adult mosquitoes, which would be impossible to use as a control measure. Previous studies have used bacteria to express dsRNA in insects to knock down the function of specific genes [119,131,132]. Ingested bacteria must be broken down by digestive enzymes or factors that disturb the bacterial cell wall and membrane in order to release the dsRNA into the insect gut [133]. Bacteria-delivered dsRNA was used to knock down the expression of *Rhodnius* haem-binding protein (RHBP) and catalase (CAT) in *R. prolixus* that negatively affected the longevity of females and the viability of their eggs [119]. This RNAi method is cheap, less labour intensive and can potentially be used for vector control.

In this chapter, we describe the amplification of a cathepsin B template for incorporation into a bacterial system that can be fed to mosquitoes with the goal of expressing dsRNA for cathepsin B in the midgut of *Ae. aegypti* to knock down the expression of this gene. After bacteria-feeding, we either blood-fed or did not blood-feed the mosquitoes before we evaluated the knockdown of cathepsin B. This is because cathepsin B is expressed at very low levels in mosquitoes that are not blood-fed; it is an inducible gene that is turned on after blood enters the midgut [134]. Silencing controls for the expression of dsRNA included bacteria that were engineered to express dsRNA that targets the aintegumenta gene from a plant, *Arabidopsis thaliana*. This control was designed to evaluate the introduction of the expression of dsRNA that does not match any mosquito genes, on the expression of cathepsin B. In addition, we determined if ingesting this modified bacterium and reducing the expression of endogenous cathepsin B would have any effects on the longevity, egg viability, and fecundity of *Ae. aegypti* females.

2.2. Methods

2.2.1. Mosquito rearing

Our laboratory colony of *Ae. aegypti* (Liverpool strain[135]) was maintained under standard laboratory conditions at 27 ± 2℃, 70% relative humidity and a 12:12 hours light:dark cycle. Eggs were laid on wet filter papers and hatched in autoclaved water. Larvae developed in water trays at a density of 100 larva/L of distilled water and were fed with crushed fish food. Adults were kept in cardboard cages with netting on top at a density of 0.5 adult/cm^3.

2.2.2. DNA extraction, total RNA extraction, and cDNA synthesis

DNA from *Ae. aegypti* and total RNA from *Arabidopsis thaliana* were extracted using TRI Reagent (Millipore Sigma, Etobicoke ON) following manufacturer's protocols. The plant gene was selected as a control for the expression of dsRNA not related to a mosquito gene. Fifty mg of tissue was used for mosquito DNA extraction and the DNA pellet was resuspended in 8 mM NaOH and stored at -20C. RNA was extracted from buds and young leaves of *A. thaliana.* RNA samples were resuspended in 25 µl of DEPC water and stored at – 80℃ until use.

Total RNA extracted from *A. thaliana* was used to synthesize cDNA using the 5X All-In-One RT MasterMix (Applied Biological Materials, Richmond BC) following manufacturer's protocols. The cDNA synthesis reaction contained 737.6 ng/µl of total RNA, 2 µl of 5X All-In-One RT MasterMix, and nuclease-free water (NFH_2O) to a final volume of 10 µl. cDNA synthesis was performed using a PTC-200 Peltier Thermal Cycler (MJ Research, Saint Bruno QC) with the settings as follows: 25℃ for 10 mins, 42℃ for 50 mins, and 85℃ for 5 mins. The cDNA was kept at – 20℃ until use.

2.2.3. Generating dsRNA constructs

A 491 bp region of the cathepsin B gene (AAEL007585) was amplified by PCR using 5 ng of *Ae. aegypti* genomic DNA as a template. PCR products were generated in 25 µl reactions containing 2.5 µl of Taq DNA polymerase buffer, 1 µl of $MgCl_2$, 1 µl of dNTPs, 1 µl of each primer (10 µM) (see cathepsin B primer sequences in Appendix A),

0.1 µl of Taq DNA polymerase (Invitrogen, Waltham MA) and 5 ng of DNA using touchdown PCR: 94℃ for 5 mins, followed by 41 cycles of 94℃ for 30 secs, an initial annealing temperature of 65℃ for 1 min and 72℃ for 45 secs, and a final elongation step at 72℃ for 5 mins. The annealing temperature was reduced by 1℃ per cycle for 11 cycles and then remained at 54℃ for 30 cycles [129]. PCR products were visualized on a 1X TAE agarose gel, and putative cathepsin B amplicons were extracted from the gel using Wizard SV Gel & PCR clean-up system (Promega, Madison WI) according to manufacturer's protocols. The eluted product was concentrated using Amicon Ultra 30K centrifugal filter (Millipore Sigma, Etobicoke ON) with 30 mins centrifugation at highest speed at room temperature according to manufacturer's protocols. The concentration of the DNA was determined using Qubit 2.0 Fluorometer (Invitrogen, Waltham MA), and the amplicon was sequenced at the NAPS Unit DNA Sequencing Facility at UBC.

Once the sequencing results confirmed the amplification of a region of the cathepsin B gene, the confirmed product was used in another PCR reaction using the same primers as used originally, to which T7 promoter overhangs were incorporated (see Appendix A for primer sequences). PCR reactions (25 µl) were prepared as described for touchdown PCR: 94℃ for 5 mins, followed by 40 cycles of 94℃ for 30 secs, an initial annealing temperature of 70℃ for 1 min and 72℃ for 45 secs, and a final elongation step at 72℃ for 5 mins. The annealing temperature was reduced by 1℃ per cycle for 10 cycles and then remained at 60℃ for 30 cycles. An aliquot of the PCR reaction was separated by electrophoresis as described above to confirm the amplification. The remaining PCR product (cathepsin B-T7) was extracted using ethanol precipitation. The PCR product was mixed with 30% volume of cold 3M sodium acetate buffer, pH 5.2. Cold 100% ethanol was added to obtain a total volume 3X the volume of the PCR product, and DNA was precipitated by incubation at 4℃ for 1 hour. The mixture was centrifuged at 12,000 x g for 15 mins at 4℃, the supernatant was discarded, and the pellet was washed with 1 volume of cold 70% ethanol. After centrifugation for 2 mins, residual ethanol was evaporated for 10 mins at room temperature, the DNA pellet was resuspended in 15 µl of NFH_2O and the concentration of extracted DNA was measured using Qubit.

An aliquot of this product was reamplified using T7 primers alone with the same touchdown PCR settings as for cathepsin B–T7 amplification. Bands were excised from the gel, and DNA was extracted using ethanol precipitation as described above, and the

product was sequenced at the NAPS Unit DNA Sequencing Facility at UBC to confirm the full incorporation of the T7 promotor regions onto our cathepsin B DNA fragment.

The protocol to obtain a template of our silencing control gene, aintegumenta (U41339.1) from *A. thaliana*, was done in a similar manner to that of the cathepsin gene. Primers to amplify a 516 base pairs (bp) region of the aintegumenta cDNA (see Table A1 for primer sequences) were used in a Safe-Green 2X PCR Taq MasterMix (Applied Biological Materials, Richmond BC). The 25 µl PCR reaction contained 12.5 µl of Safe-Green 2X PCR Taq MasterMix, 1 µl of each primer (10 µM) and 1 µl of *A. thaliana* cDNA, under the following conditions: 94℃ for 5 mins, followed by 35 cycles of 94℃ for 30 secs, 53℃ for 30 secs and 72℃ for 45 secs, and a final elongation step at 72℃ for 5 mins. The products of the PCR amplification, were extracted from the gel, purified, reused to incorporate the T7 overhangs using PCR, and were sequenced as was described for the cathepsin B product. Our aintegumenta sequence was blasted against *Ae. aegypti* genome to make sure that the dsRNA would not target any mosquito genes.

2.2.4. Cloning, plasmid Isolation, and transformation

The DNA constructs for cathepsin B and aintegumenta containing the T7 extensions were ligated into pGEM-T plasmids (Promega, Madison WI) in 10 µl ligation reactions containing 5 µl of 2X Rapid Ligation Buffer, 1 µl of the pGEM-T vector, 8.3 ng of PCR product and 1 µl of T4 DNA Ligase. Reactions were mixed via pipetting and incubated overnight at 4℃. The plasmids were transformed into JM109 High Efficiency Competent Cells (Promega, Madison WI). JM109 High Efficiency Competent Cells were thawed on ice, and added to 2 µl of each ligation reaction. These tubes were placed on ice for 20 mins, heat-shocked for 45 secs at 42℃ in a water bath and immediately placed on ice for 2 mins. Next, 500 µl of Luria–Bertani (LB) broth was added to the reactions, followed by 1.5 hrs incubation at 37℃, 150 rpm. Finally, 100 µl or 250 µl of each transformation culture was plated onto LB/ ampicillin/ IPTG/ X-Gal plates and incubated overnight at 37℃.

Colony PCR was used to screen the putative transformants by inoculating standard 25 µl PCR reactions with a small portion of selected bacterial colonies. PCR reactions contained 12.5 µl Safe-Green 2X PCR Taq MasterMix and 1 µl of each primer (10 µM). Cathepsin B PCR settings were as follows: 94℃ for 5 mins, 35 cycles of 94℃

for 30 secs, 54℃ for 30 secs and 72℃ for 30 secs, followed by 72℃ for 5 mins. The PCR conditions to amplify the aintegumenta amplicon were: 94℃ for 5 mins, followed by 35 cycles of 94℃ for 30 secs, 53℃ for 30 secs and 72℃ for 45 secs, and a final elongation step at 72℃ for 5 mins. The PCR products were separated on 1X TAE agarose gels. Once colony PCR identified putative transformants, the remaining portion of each bacterial colony was inoculated into 5 mL of LB + ampicillin, grown overnight, and these cultures were used to make streak plates and glycerol stocks. The remainder of the overnight growth was used to purify plasmids using QIAprep Spin Miniprep Kit (Qiagen, Hilden NW) according to manufacturer's protocols. Plasmids were eluted in 30 µl of NFH_2O. These plasmids were evaluated again using PCR and sequenced to confirm they contained the amplicon of interest.

The isolated plasmids were transformed into *Escherichia coli* HT115 (DE3) as described [136]. HT115 (DE3) cells were first made competent: Cells were grown overnight in LB broth containing 12.5 µg/ml of tetracycline. The overnight culture was diluted 100-fold in LB broth containing tetracycline and grown to an OD_{600} of 0.4 via shaking at 37℃, 180 rpm. The cells were chilled on ice for 5 mins, and harvested via centrifugation for 10 mins at 4℃, 3000 rpm. The cells were resuspended in 1 ml of cold 50 mM $CaCl_2$ and kept on ice. For transformation, 1 µl of plasmid was added to 100 µl of competent cells and incubated on ice for 30 mins. The transformation culture was then heat-shocked for 1 min at 37℃ and kept on ice for 2 mins. 1 ml of LB broth was added to each tube and the mixtures were incubated at 37℃, 150 rpm for 1 hr. Aliquots of the transformation culture were plated on LB plates containing 100 µg/ml of ampicillin and 12.5 µg/ml of tetracycline and grown overnight at 37℃. Colony PCR reactions were done on putative transformants and PCR products were sequenced at the NAPS Unit DNA Sequencing Facility at UBC.

2.2.5. Assessment of cathepsin B expression

Preparation of bacteria for feeding

Escherichia coli HT115 (DE3) engineered to express dsRNA that targets cathepsin B (HT115-CAT) and aintegumenta (HT115-ANT) was grown in LB media containing 50 µg/ml of ampicillin and 12.5 µg/ml of tetracycline overnight at 37℃, 200 rpm. For untransformed *E. coli* HT115 (DE3) that does not express dsRNA (HT115-X),

ampicillin was not added to the LB media. Overnight cultures were diluted to OD_{600} of ~0.1 in Yeast Extract Tryptone (2X YT) media containing antibiotics, grown to OD_{600} of 0.4 – 0.6, and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 2 mM. IPTG was not added to the uninduced controls (uninduced HT115-CAT). After 4 hours of induction at 37℃ at 200 rpm, bacteria were harvested by centrifugation at 4,400 x g for 10 mins at 4℃ and resuspended with 10% sterile sucrose to a concentration of 1×10^{10}cfu/ml.

We confirmed that dsRNAs were expressed by these bacteria. Total RNA was extracted from 1 ml of the induced HT115-CAT and HT115-ANT cultures using TRI Reagent following manufacturer's protocols. One volume of phenol (pH 4) was added, vortexed for 3 mins, and centrifuged at 12,000 x g for 5 mins at room temperature. The aqueous layer was mixed with 8 volumes of chloroform-isoamyl alcohol (24:1), centrifuged again to obtain the aqueous layer. Double-stranded RNA was precipitated in 0.75 volumes of isopropanol by 3 cycles of freezing and thawing with liquid Nitrogen, centrifuged at 12,000 x g for 5 mins at 4℃, washed with 75% ethanol, centrifuged again, and residual ethanol was evaporated for 10 mins at room temperature. The resultant the pellet was resuspended in 15 µl of NFH_2O and run on a bleach gel to check for the presence of dsRNA.

Bacteria-feeding and blood-feeding

Females were fed with induced HT115-CAT, uninduced HT115-CAT, induced HT115-ANT, uninduced HT115-X or no bacteria. Bacteria were mixed with 10% sugar solution (1×10^{10}CFU/ml) and cotton balls soaked with the sugar-bacteria mixture were accessible to the mosquitoes *ad libitum.* These solutions were replaced daily. Subsequently, 3 days after first exposure to the bacteria in the sucrose solution, mosquitoes were exposed to sheep's blood for 20 mins and allowed to feed to repletion. Blood-feeding was done in a water jacketed membrane feeder in which blood was kept at 37℃ and the mosquitoes feed on the blood by piercing a stretched piece of parafilm with their proboscises. After feeding, 30 engorged mosquitoes were transferred to new cages with access *ad libitum* to a 10% sucrose solution until midgut dissections were performed.

Midgut dissection and total RNA extraction

Midgut dissections were conducted at 5 hrs (day 1), 53 hrs (day 3), 101 hrs (day 5) post blood-feeding. Mosquitoes were surface sterilized with 70% ethanol, and then rinsed twice with sterile water. The midguts of mosquitoes were then dissected on a drop of sterile PBS. Dissected midguts were washed with cold DEPC-PBS to remove the bloodmeal. Some midguts were also stored at -80℃. At each time point, 7 midguts were pooled for total RNA extraction using 200 µl of TRI Reagent following manufacturer's protocols. The extracted RNA was resuspended in DEPC water, quantified using a NanoDrop 2000C (ThermoFisher Scientific, Waltham MA), and stored at – 80℃.

DNase treatment and cDNA synthesis

Total RNA (1 µg) was treated with DNase in a 10 µl reaction consisting of 1 µl of DNase I, RNase-free Kit (ThermoFisher Scientific, Waltham MA) and 1 µl of 10X reaction buffer. After incubation at 37℃ for 30 mins, DNase was inactivated by adding 1 µl of 50 mM EDTA, followed by incubation at 65℃ for 10 mins.

Total RNA (100 ng) from each treatment group was reverse transcribed in 20 µl reactions containing 4µl of 5X All-In-One RT MasterMix. The cDNA synthesis settings were as follows: 65℃ for 5 mins, 42℃ for 50 mins and 70℃ for 15 mins. The success of cDNA synthesis was verified by amplifying a constitutively expressed gene, actin-5 (LOC5574526), with standard PCR (see Appendix A for primer sequences). The PCR was done in 25 µl reactions containing 12 µl Safe-Green 2X PCR Taq MasterMix ,1 µl of each primer (10 µM) and 1 µl of cDNA, with the following settings: 95℃ for 2 mins, 40 cycles of 95℃ for 10 secs, 55℃ for 10 secs and 72℃ for 30 secs. A no template control was included in each verification test.

Real-Time Quantitative PCR (qPCR) evaluation of gene expression

Real-Time Quantitative PCR (qPCR) was used to determine the relative expression of cathepsin B in midguts of treated mosquitoes. qPCR reactions were performed on a Rotor-Gene 3000 (Corbett Research, Mortlake NSW) using the PerfeCTa SYBR Green SuperMix (Quanta Biosciences, Gaithersburg, MD). The 12 µl reactions contained 6 µl of PerfeCTa SYBR Green SuperMix, 1 µl of each primer (10 µM) and 1 µl of cDNA that was diluted to 15 ng/µl with NFH_2O (see qPCR-cathepsin B primer sequences in Appendix A). A no template control was also included. All samples

were run in duplicate using the PCR settings: 95℃ for 2 mins, followed by 40 cycles of 95℃ for 10 secs, 50℃ for 15 secs and 72℃ for 20 secs. β-actin was used as a reference housekeeping gene for normalization (see qPCR-β-actin primer sequences in Appendix A). Data were analyzed using the $2^{\Delta CT}$ and $2^{-\Delta\Delta CT}$ to calculate relative expression levels [137]. A cutoff value of 1.71-fold change was determined in our previous microarray data by conducting self–self hybridization, and thus a fold change greater than this cutoff value was considered to be statistically significant [111]. For this knockdown experiment, we increased the cutoff value to 2-fold change. Nevertheless, statistical significance is not the same as biological significance, because in some cases, a statistically insignificant change in gene expression could be sufficient to significantly affect the phenotype of the organism. In our previous knockdown study, a 1.33 -fold change in cathepsin B in Rockefeller mosquitoes could significantly decrease the prevalence of DENV-2 infection in the mosquitoes [111]. Melt curve analysis was done to check for nonspecific amplification.

In subsequent repeats, our basic protocols were modified:

1) RNaseOFF Ribonuclease Inhibitor (Applied Biological Materials, Richmond BC) was added to each RNA sample to a final concentration of 1 U/µl before DNase treatment to protect RNA from degradation.

2) TURBO DNA-free Kit (ThermoFisher Scientific, Waltham MA) was used for DNase treatment instead of DNase I, RNase-free Kit. DNase-treated RNA was checked for integrity and DNA contamination before performing cDNA synthesis.

3) An RNA integrity test was performed on the DNase-treated RNA samples by running 100 ng of RNA on a bleach gel. The gel apparatus was sprayed with RNaseZap RNase Decontamination Solution (ThermoFisher Scientific, Waltham MA), left for 5 mins and then rinsed with distilled water. To prepare a bleach gel, 1.0% w/v agarose and 1.0% w/v of Clorox bleach were mixed with 1X TAE buffer. RNA loading buffer was added to each sample before loading on the bleach gel. The gel was run at 60 V for 80 mins and checked for degradation.

4) The DNase-treated RNA samples were used in PCR reactions to evaluate DNA contamination. Each 25 µl PCR reaction contained 12 µl of Safe-Green 2X PCR Taq MasterMix and 1 µl of each primer pair (10 µM) (see qPCR-β-actin or qPCR-cathepsin B primers in Appendix A). Positive bands indicated DNA contamination of the RNA.

5) After confirming the RNA integrity and lack of DNA contamination, 400 ng of total RNA was reverse transcribed in each cDNA synthesis reaction as described above. One µl of cDNA was used for each qPCR reaction. qPCR was done as previously described, but a LightCycler 96 (Roche, Mississauga, ON) was used instead of Rotor-Gene 3000.

2.2.6. Fitness tests

We tested whether the ingestion of bacteria and the potential effect of knocking down the expression of cathepsin B would affect the fecundity, egg viability, and longevity of adults. *Aedes aegypti* females were fed with induced HT115-CAT for 3 days. Meanwhile, control groups were either fed with uninduced HT115-CAT or 10% sucrose solution only (no bacteria). After bacteria-feeding, mosquitoes were starved overnight and fed with sterile sheep's blood on the next day. Throughout these tests, mosquitoes were fed with 10% sucrose on a daily basis under standard laboratory conditions at 27 ± 2℃, 70% relative humidity and a 12:12 hours light:dark cycle. The protocols for sugar, bacteria or blood-feeding were previously described. All tests were repeated for 3 times. In each repeat, there were 10 females per treatment group for fecundity and longevity tests, and 20 eggs from each of the 10 females per treatment group for egg viability test.

Fecundity

Three days after blood-feeding, 10 engorged females from each treatment group were transferred individually into oviposition tubes. Each oviposition tube contained a piece of filter paper placed on top of a cotton ball that was partially submerged in distilled water. The rearing conditions were 27 ± 2℃, 70% relative humidity and a 12:12 hours light:dark cycle. The eggs from each female were counted after 3 days.

Egg viability

We assessed the viability of eggs from the same females that undergone fecundity test. One week after oviposition, 20 eggs from each female were transferred into a tube containing 30 ml of autoclaved water and a pinch of crushed fish food. 4 days later, larvae were counted to determine the proportion of eggs that hatched. The rearing conditions were the same as above.

Longevity

We investigated whether any of our bacterial constructs affected the longevity of mosquitoes, with or without blood-feeding. After bacteria and blood-feeding, 10 mosquitoes from each treatment group were transferred into a cage containing a strip of paper towel and scored for lifespan by each day they survived. Three days after blood-feeding, the paper towels were moistened with distilled water to allow the mosquitoes to oviposit. For longevity test without blood-feeding, there was no paper towel in the cages. The rearing conditions were the same as above.

Statistical analysis

All analyses were done using JMP statistical software 13.1.0 (SAS Institute). For fecundity and longevity tests, the means were compared using least square means analysis. Repeats were treated as random effects to account for their variability. For the egg viability test, the proportions of eggs that hatched were compared using a generalized linear model fit analysis with a binomial distribution and a logit link. In this analysis, we also used an overdispersion test and Firth's bias-adjusted estimates.

2.3. Results

2.3.1. Assessment of cathepsin B expression

All constructs were amplified, cloned, and then sequenced to ensure that they were expressing dsRNA to knock down the expression of cathepsin B, or to express dsRNA complementary to a plant gene as a control (see Appendix B for the sequencing result of cathepsin B and aintegumenta). We had also blasted our aintegumenta sequence against the genome of *Ae. aegypti*, and there was no significant match. The production of dsRNA in induced HT115-CAT and HT115-ANT was confirmed by

performing dsRNA extraction and visualizing the dsRNA on a gel (see Appendix B for gel image). Mosquitoes fed on the bacteria in the sugar solution, and then fed well on the bloodmeal 24 hours later.

We first tested the effect of the different treatments on cathepsin B expression in the midguts of *Ae. aegypti* females prior to blood-feeding (see Appendix B for representative qPCR amplification and melt curves for cathepsin B and β-actin). There was a significant reduction in the expression of cathepsin B in the mosquitoes that ingested bacteria engineered to express dsRNA to target cathepsin B (HT115-CAT) compared with mosquitoes that ingested no bacteria (Figure 2.1). When we compared the expression of cathepsin B in mosquitoes fed bacteria expressing the dsRNA plant gene control (HT115-ANT), we also see a reduction in expression of cathepsin B, relative to the expression in no bacteria controls except at day 5 in which we see a similar level of expression in HT115-ANT fed and no bacteria controls (Figure 2.2). Mosquitoes that received no bacteria had higher cathepsin B expression than those that ingested bacteria containing no plasmids (HT115-X), except for day 5 (Figure 2.3). Mosquitoes that ingested HT115-CAT bacteria had a ~75% knockdown effect at day 7, but no significant knockdown in between days 1-5 compared with mosquitoes that had ingested HT115-ANT (Figure 2.4).

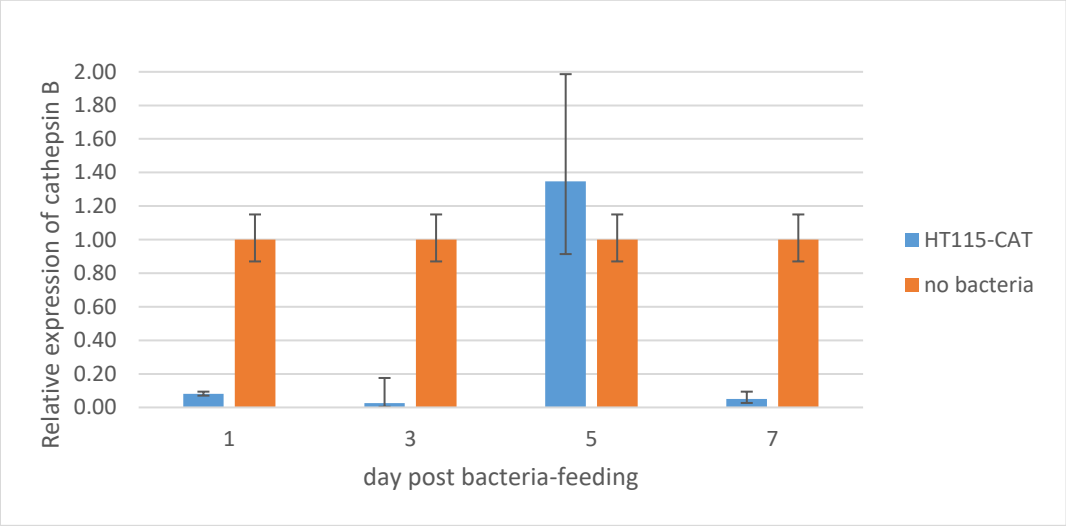

Figure 2.1 **Comparative expression of cathepsin B in the midguts of *Aedes aegypti* females fed with HT115-CAT (blue bars) compared with females fed with no bacteria (orange bars). The no bacteria controls were arbitrarily set at 1 and expression levels in the HT115-CAT represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

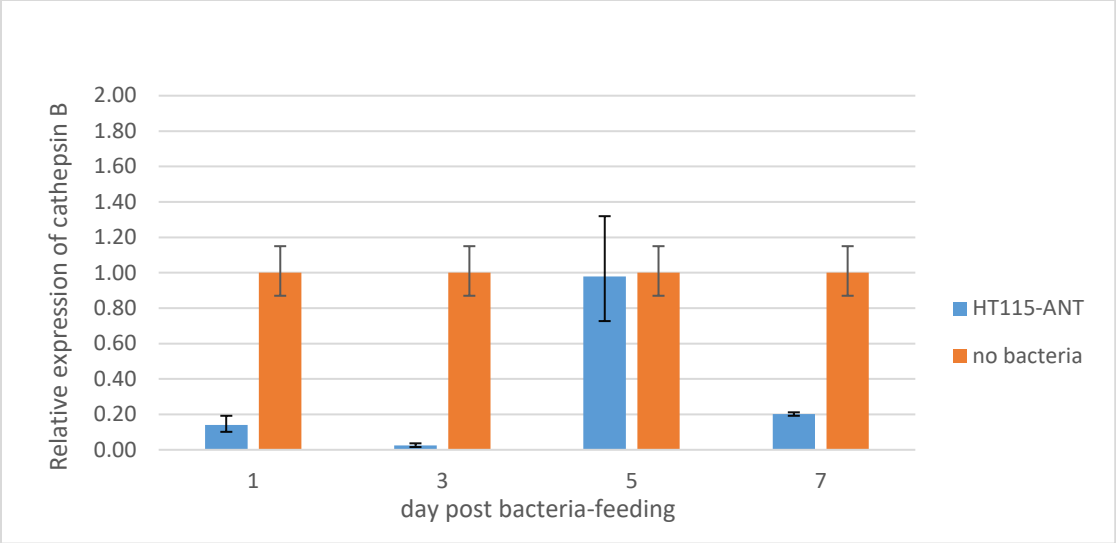

Figure 2.2 **Comparative expression of cathepsin B in the midguts of *Aedes aegypti* females fed with HT115-ANT (blue bars) compared with females fed with no bacteria controls (orange bars). The no bacteria controls were arbitrarily set at 1 and expression levels in the HT115-ANT represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

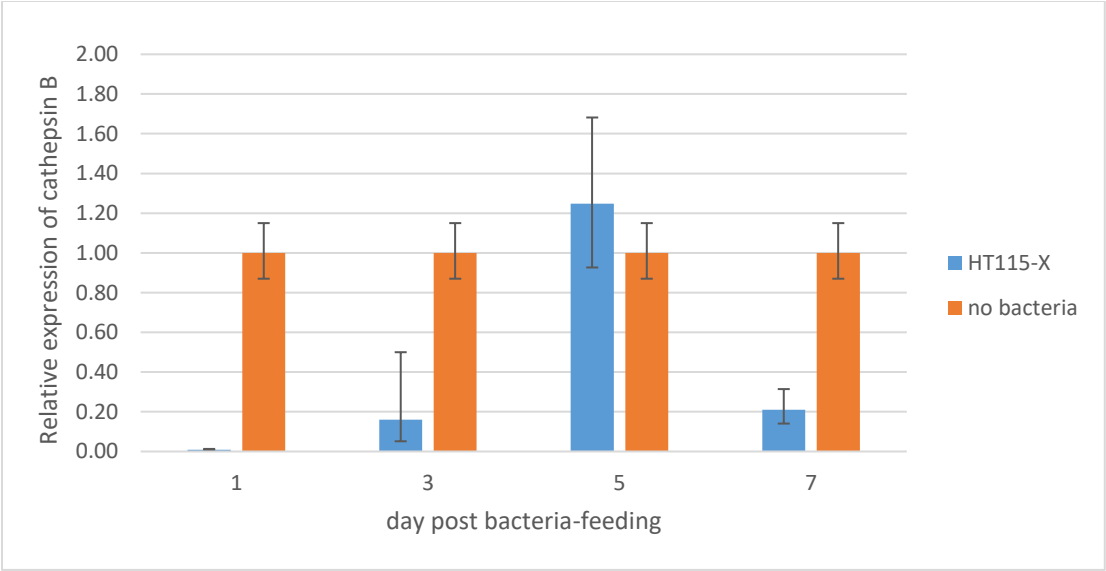

Figure 2.3 **Comparative expression of cathepsin B in the midguts of *Aedes aegypti* females fed with no plasmid controls, HT115-X (blue bars) compared with females fed with no bacteria controls (orange bars). The no bacteria controls were arbitrarily set at 1 and expression levels in the HT115-X represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

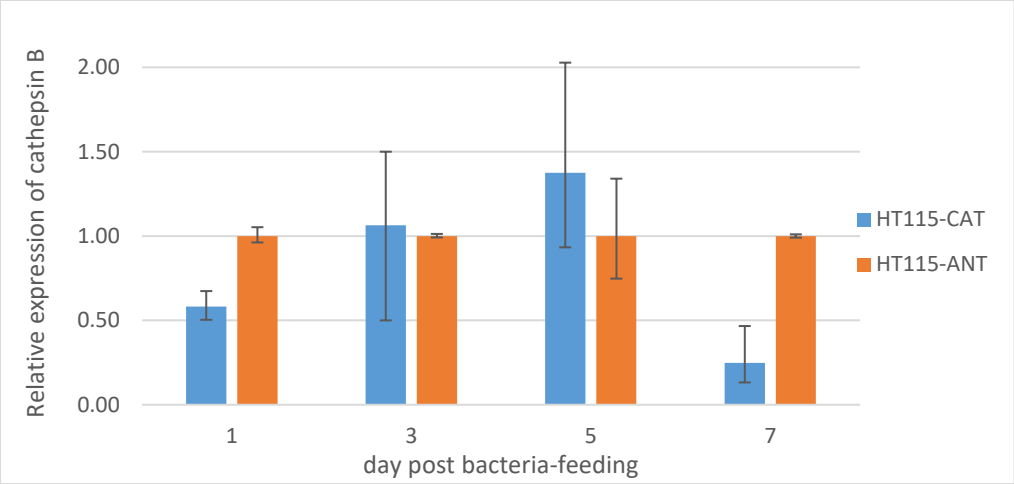

Figure 2.4 **Comparative expression of cathepsin B in the midguts of *Aedes aegypti* females fed with HT115-CAT (blue bars) or HT115-ANT constructs (orange bars). Expression levels in the HT115-ANT treated mosquitoes were arbitrarily set at 1 and expression levels in the HT115-CAT represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

We then fed mosquitoes on the bacterial constructs and then exposed them to a bloodmeal, which should increase the expression of cathepsin B. When we compare the expression levels of cathepsin B in mosquitoes blood-fed after ingesting bacteria expressing the cathepsin B knockdown construct (HT115-CAT) compared with no bacteria controls, we find inconsistent results. Expression levels in the HT115-CAT mosquitoes were reduced by 50% at day 1 and did not differ significantly at days 3-7 (Figure 2.5). When we fed mosquitoes with no bacteria or uninduced HT115-CAT constructs we see a ~60% reduction in cathepsin B expression at day 1, but no significant differences on other days (Figure 2.6). When we compare expression in mosquitoes that ingested induced or uninduced HT115-CAT constructs prior to blood-feeding, we see no significant differences (Figure 2.7). When we compared the expression of cathepsin B in mosquitoes that received the constructs expressing dsRNA for cathepsin B (HT115-CAT) or the construct to knock down a plant gene (HT115-ANT) before feeding on blood, we see a 100% increase in the expression of cathepsin B in mosquitoes that ingested HT115-CAT at day 1, a 400% increase at day 3, and a 60% decrease at day 5 (Figure 2.8). In comparisons of cathepsin B expression in mosquitoes fed on induced or uninduced constructs of HT115-CAT we see no significant difference at day 1, a 60% reduction in cathepsin B expression in mosquitoes that fed on the

induced constructs at day 3, but an overexpression in HT115-CAT fed mosquitoes at day 5 (Figure 2.9).

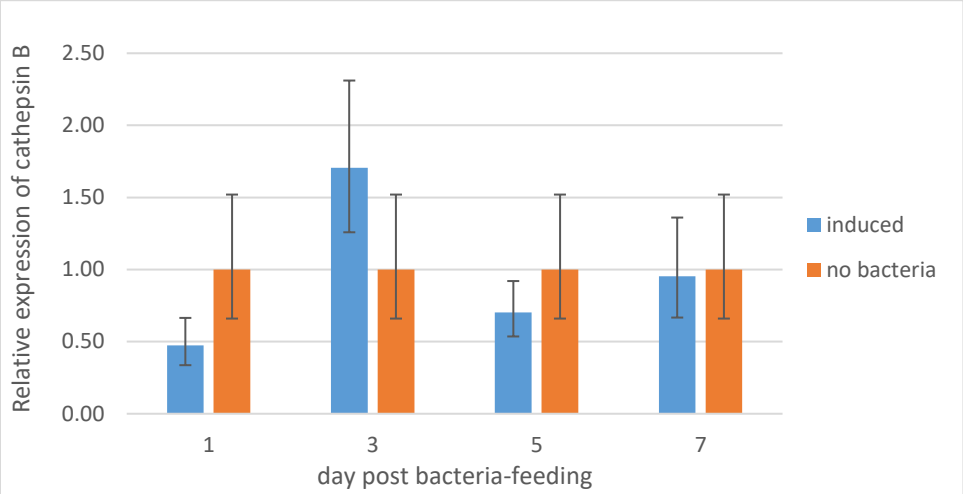

Figure 2.5 **Comparative expression of cathepsin B in the midguts of *Aedes aegypti* females fed with induced HT115-CAT (blue bars) or no bacteria (orange bars) and then fed on blood. Expression levels in the no bacteria controls were arbitrarily set at 1 and expression levels in the induced HT115-CAT represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

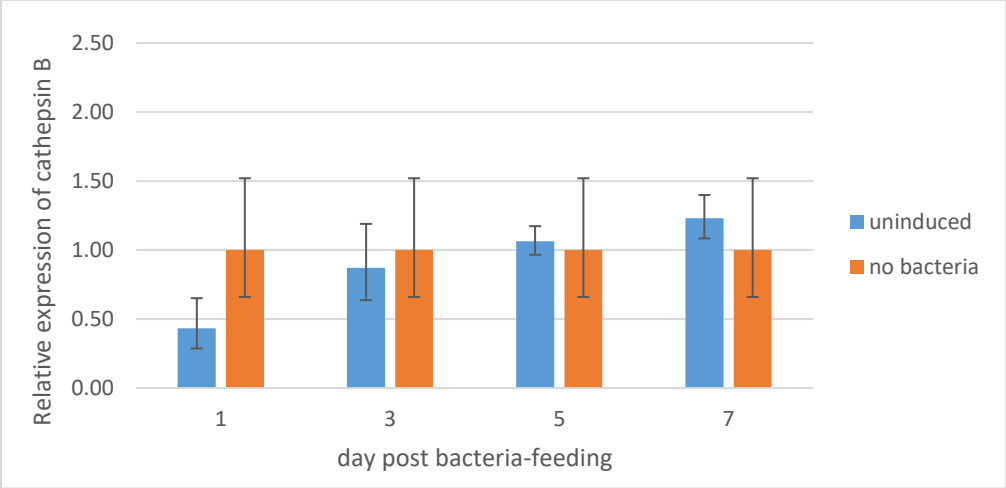

Figure 2.6 **Comparative expression of cathepsin B in the midguts of *Aedes aegypti* females fed with uninduced HT115-CAT (blue bars) or no bacteria (orange bars) and then fed on blood. Expression levels in the no bacteria controls were arbitrarily set at 1 and expression levels in the uninduced HT115-CAT represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

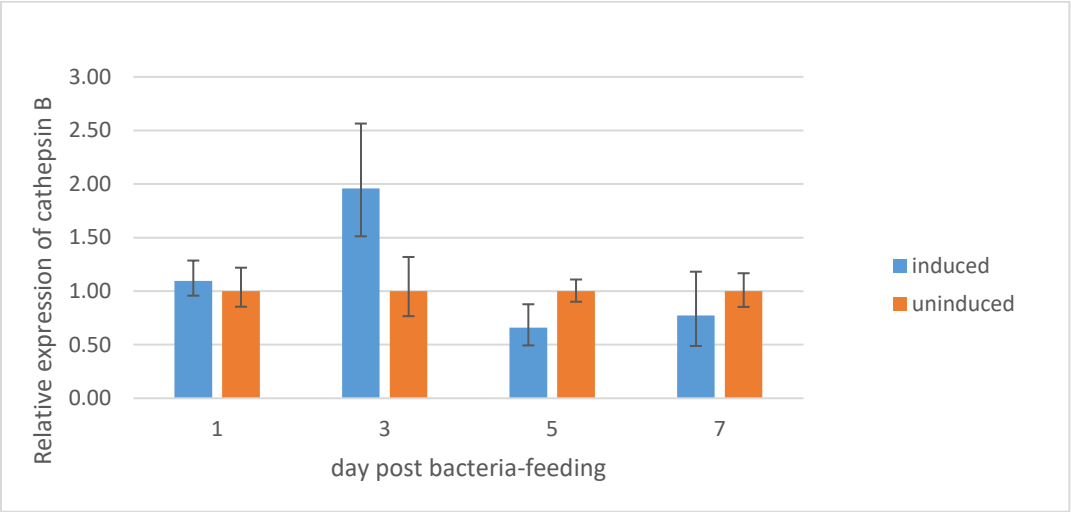

Figure 2.7 **Comparative expression of cathepsin B in the midguts of *Aedes aegypti* females fed with uninduced HT115-CAT control (orange bars) or induced HT115-CAT constructs (blue bars), and then fed on blood. Expression levels in the uninduced constructs were arbitrarily set at 1 and expression levels in the induced HT115-CAT represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

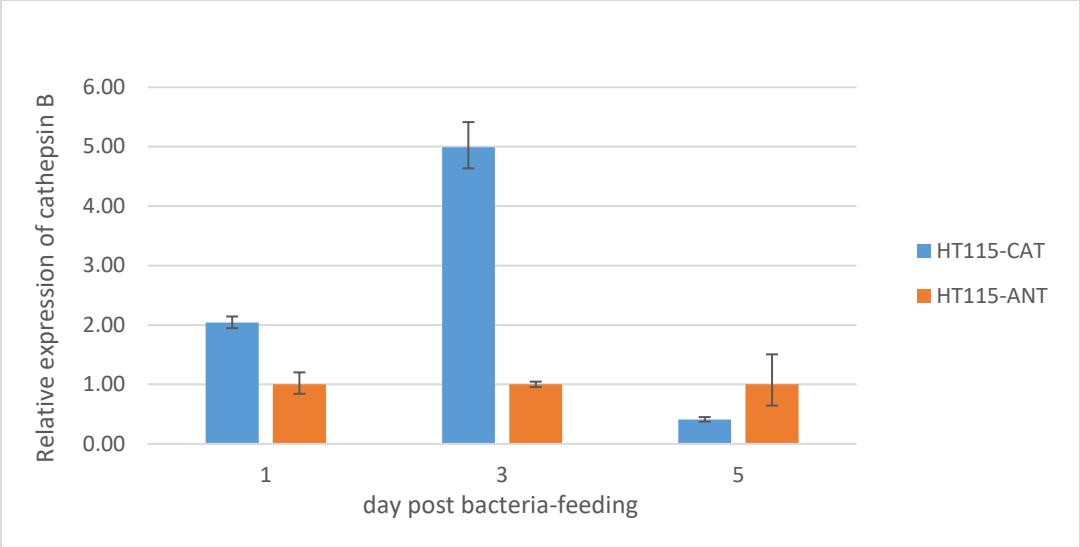

Figure 2.8 **Comparative expression of cathepsin B in the midguts of *Aedes aegypti* females fed with HT115-ANT (orange bars) or induced HT115-CAT constructs (blue bars), and then fed on blood. Expression levels in the HT115-ANT constructs were arbitrarily set at 1 and expression levels in the induced HT115-CAT represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

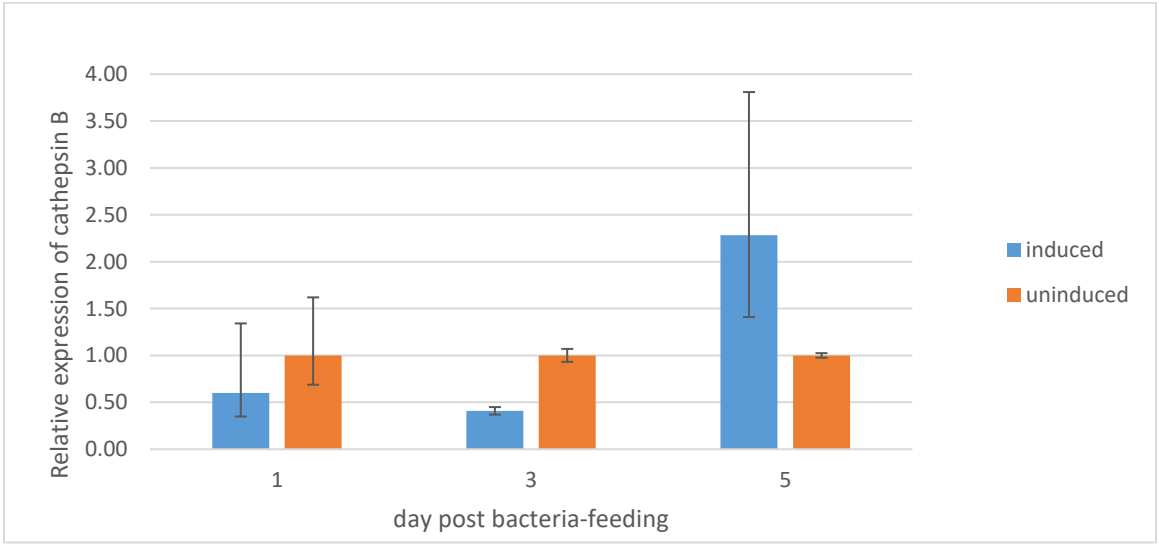

Figure 2.9 **Comparative expression of cathepsin B in the midguts of *Aedes aegypti* females fed with induced HT115-CAT (blue bars) or uninduced HT115-CAT constructs (orange bars), and then fed on blood. Expression levels in the uninduced HT115-CAT constructs were arbitrarily set at 1 and expression levels in the induced HT115-CAT represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

2.3.2. Fitness tests

Fecundity test

Table 2.1 shows the mean number of eggs per female in each treatment group, along with the standard deviation. There were no significant differences between the number of eggs laid by *Ae. aegypti* females that had ingested induced HT115-CAT, uninduced HT115-CAT or the no bacteria control (least square means analysis, DF = 2, F Ratio = 0.40, P-value = 0.69). Details about the analysis were shown in Appendix C.

Table 2.1 The mean number of eggs laid by individual *Aedes aegypti* females fed with 10% sucrose solution that contained HT115-CAT bacteria engineered to knock down the expression of cathepsin B. No bacteria controls were fed with 10% sucrose solution only, whereas uninduced controls were fed with uninduced HT115-CAT in 10% sucrose solution. The number of eggs represents the mean of 3 repeats, n = 10 per repeat.

Condition	The number of eggs per female	Standard deviation
HT115-CAT	80.8	14.6
No bacteria control	75.8	36.4
Uninduced control	73.3	32.3

Egg viability test

Table 2.2 demonstrates the mean proportion of egg hatched per female in each treatment group, with the confidence interval. There were no significant differences in the percentage of eggs that hatched when the eggs were laid by *Ae. aegypti* females treated with induced HT115-CAT, uninduced HT115-CAT, or no bacteria controls (generalized linear model fit analysis with a binomial distribution and a logit link, DF = 2, ChiSquare = 0.10, P-value = 0.95). Bacteria-delivered dsRNA of cathepsin B did not affect egg viability of *Ae. aegypti*. Details about the analysis were shown in Appendix D.

Table 2.2 **The proportion of eggs hatched from *Aedes aegypti* females fed with 10% sucrose solution that contained HT115-CAT bacteria engineered to knock down the expression of cathepsin B. No bacteria controls were fed with 10% sucrose solution only, whereas uninduced controls were fed with uninduced HT115-CAT in 10% sucrose solution. The proportion of eggs hatched represents the mean of 3 repeats, n = 10 per repeat, 20 eggs per female.**

Condition	The proportion of eggs hatched	Lower 95% mean	Upper 95% mean
HT115-CAT	0.38	0.05	0.86
No bacteria control	0.42	0.07	0.88
Uninduced control	0.30	0.03	0.84

Longevity test

Table 2.3 shows the longevity test results. There were no significant differences in longevity of *Ae. aegypti* females fed with induced HT115-CAT, uninduced HT115-CAT or no bacteria controls with blood-feeding (least square means analysis, DF =2, F Ratio = 3.11, P-value = 0.15) or without blood-feeding (least square means analysis, DF = 2, F Ratio = 0.67, P-value = 0.56). After pooling the longevity data for with and without blood-feeding, there was no significant difference between the females treated with induced HT115-CAT, uninduced HT115-CAT or no bacteria controls (least square means analysis, DF =2, F Ratio = 3.06, P-value = 0.08). Mosquitoes that blood-fed and oviposited had shorter lifespans than those that did not blood-feed (least square means analysis, DF = 1, F Ratio = 5.76, P-value = 0.03). Bacteria-feeding had no interaction with blood-feeding (least square means analysis, DF = 2, F Ratio = 0.13, P-value = 0.88). Details about the analysis were shown in Appendix E.

Table 2.3 The longevity of *Aedes aegypti* females fed with 10% sucrose solution containing HT115-CAT bacteria engineered to knock down the expression of cathepsin B, with or without blood-feeding. No bacteria controls were fed with 10% sucrose solution only, whereas uninduced controls were fed with uninduced HT115-CAT in 10% sucrose solution. The lifespan represents the mean of 3 repeats, n = 10 per repeat.

Condition		Lifespan (days)	Standard deviation
Not blood-fed	HT115-CAT	36.1	7.4
	No bacteria control	40.2	8.6
	Uninduced control	37.5	7.4
	Mean	37.9	7.9
Blood-fed and oviposited	HT115-CAT	32.2	5.3
	No bacteria control	37.4	8.0
	Uninduced control	32.5	6.0
	Mean	34.0	6.9

2.4. Discussion

Cathepsin B is expressed at very low levels in mosquitoes that are not blood-fed; it is an inducible gene that is turned on after blood enters the midgut [134]. Our results demonstrate that the expression of cathepsin B is very variable, and we were unable to knock down the expression of cathepsin B significantly by feeding mosquitoes on bacteria that were engineered to express dsRNA to knock down cathepsin B expression. On many occasions, we could not get a good signal using qPCR in bacteria-fed insects.

Prior to blood-feeding, we saw conflicting data. Sometimes, cathepsin B expression was knocked down by ~80-90% at days 1, 3 and 7 in mosquitoes that ingested HT115-CAT bacteria engineered to express dsRNA for cathepsin B compared with no bacteria controls (Figure 2.1) but this knockdown was also seen in mosquitoes that had fed on HT115-ANT bacteria engineered to express dsRNA for a plant gene (Figure 2.2) or HT115-X that are not engineered to express any dsRNA (Figure 2.3). Equivalent knockdowns of cathepsin B in mosquitoes fed with HT115-CAT, HT115-ANT, and HT115-X were unexpected. After blood-feeding we measured a knockdown on cathepsin B expression in insects that ingested HT115-CAT at days 1, but not at days 3-7 (Figure 2.5) compared with no bacteria controls. We also saw a 60% decline in cathepsin B expression in mosquitoes that ingested uninduced HT115-CAT bacteria compared with no bacteria controls at day 1, but not at days 3-7 (Figure 2.6). However,

when we fed mosquitoes the HT115-CAT or the HT115-ANT constructs, we actually found a higher relative expression in the HT115-CAT fed group (Figure 2.8).

We were not able to knock down the expression of cathepsin B in a predictable and reliable manner using a bacteria delivery system that has been used previously in a different insect vector, *Rhodnius prolixus*, although that system has stopped working reliably [119]. Several genes that were knocked down successfully using microinjection of dsRNA had minimal or no knockdown with bacterially mediated RNAi in mosquitoes and other insects [118,138]. From an engineering perspective, bacteria-delivered dsRNA is less effective than microinjection [118]. In the bacteria-delivered dsRNA system, bacteria must be broken down by digestive enzymes or factors that disturb the bacterial cell wall and membrane in order to release the dsRNA into the insect gut [133]. Thus, the bacteria-delivered dsRNA system has a physical hindrance of the bacterial cell wall, unlike the microinjection system in which dsRNA is directly injected into the hemolymph of the insects. Another factor is the variability in the processing of dsRNA into siRNA between the two RNAi systems, as siRNA was detected in the total RNA isolated from *Ae. aegypti* injected with dsRNA, but not in those that were fed with dsRNA [138]. Increases in expression in bacteria-fed insects could reflect the role of cathepsin B in Toll-like receptor signalling which activates components of the innate immune system in insects [139]. The bacteria-delivered dsRNA system may fail if the mosquitoes did not ingest enough bacteria, or if the bacteria did not produce enough dsRNA. We can measure the amount of ingested bacteria by performing mosquito midgut dissections, followed by colony forming unit assay using LB plates containing ampicillin and tetracycline. We can also increase the dsRNA production yield increasing the IPTG induction.

RNA quality was variable during the repeats and we worked to ensure DNA contamination did not affect our results. All cDNAs were used to amplify housekeeping gene (actin-5) to ensure that the RNA extraction and cDNA synthesis was successful. However, low expression levels of an inducible gene such as cathepsin B, in the absence of the inducer (blood) makes qPCR more variable. Even with the blood-feeding however, there were problems in the consistency of our results. This may be a factor of how many bacteria are required to express dsRNA to levels that will have a significant knockdown effect, as we could not determine precisely how many bacteria were ingested.

Cathepsin B is involved in the degradation of host hemoglobin [130]. It also degrades vitellogenin and vitellin for the production of eggs [50]. Hence, we predicted that mosquitoes treated with bacteria-delivered dsRNA cathepsin B would have reduced fecundity. Our results, however, indicated that there were no significant differences in the fecundity of *Ae. aegypti* treated with induced HT115-CAT, uninduced HT115-CAT or no bacteria. The mean number of eggs per female of mosquitoes treated with induced HT115-CAT was slightly larger, but not significantly different from, uninduced and no bacteria controls (Table 2.1). There are possible explanations: 1) the knockdown of cathepsin B in mosquitoes via bacteria-delivered dsRNA did not affect the fecundity of treated mosquitoes, 2) the level of cathepsin B knockdown was not sufficient to affect the fecundity of treated mosquitoes. We could determine the level of knockdown required to affect fecundity by microinjecting dsRNA cathepsin B, but this would defeat the purpose of developing a bacteria-delivered dsRNA strategy [17]. Our data suggest that that bacteria-fed mosquitoes, expressing dsRNA for cathepsin B had a similar fecundity to no bacteria controls. Feeding mosquitoes with a high concentration of *E. coli* (1×10^{10} CFU/ml) did not affect the fecundity of mosquitoes.

As with many similar studies, there is a large standard deviation in the fecundity data. The number of eggs produced by a female mosquito is a function of body size, nutrient reserves obtained as a larva, and the volume of blood ingested [54,140]. In our experiments, we only selected females that had engorged fully on the bloodmeal. However, there may have been variation in bloodmeal volume ingested due to differences in female size. Larger females consume more blood than smaller females, which results in a significantly higher fecundity [54,140]. Large females also could transfer more of their lipid reserves to their ovaries even before blood-feeding [141]. Furthermore, large females are more efficient in utilizing their bloodmeal for yolk synthesis [140]. As a result, the fecundity of large females is generally higher than that of smaller females. We could improve our protocol by using a fixed female size.

Our data suggest that the ingestion of bacteria, whether they expressed dsRNA or not, had no effect on the egg viability of *Ae. aegypti* (Table 2.2). There were no significant differences in the percentage of eggs that hatched among the treatment groups that were fed with induced HT115-CAT, uninduced HT115-CAT or no bacteria. We do not know if this was a result of an insufficient level of knockdown of cathepsin B, or the fact that any eggs produced with sufficient lipid, carbohydrate and protein should

survive and hatch. The range of egg hatching success in our experiment was extremely large. All such data can be improved by having larger sample sizes. Larger females, when mated with large or small males, produce eggs with a higher hatching percentage compared with smaller females mated with either large or small males [54,142].

There were no significant differences in longevity among groups of *Ae. aegypti* females treated with induced HT115-CAT, uninduced HT115-CAT or no bacteria, with or without blood-feeding and oviposition. Mosquitoes that had blood-fed and oviposited had significantly shorter lifespans than mosquitoes that did not blood-feed (Table 2.3). It is unlikely that blood-feeding itself adversely affects the longevity of mosquitoes. The lifespan of mosquitoes that fed on blood and sugar were longer than mosquitoes that fed solely on sugar, and mosquitoes fed on blood alone had no significant difference in longevity compared with mosquitoes that fed on sugar [143,144]. Females can synthesize glycogen and lipids from vertebrate blood, although this may reduce the reserves available for the eggs [42].

2.5. Conclusions

We generated bacteria to express dsRNA corresponding to cathepsin B or aintegumenta in *E. coli* HT115 (DE3) cells. Bacteria-delivered dsRNA was not able to knock down gene expression as effectively and predictably as when the dsRNA is microinjected directly into the insect. As a result, the expression of cathepsin B using bacteria-delivered dsRNA is very variable. Bacteria-delivered dsRNA of cathepsin B, however, did not affect the fitness (fecundity, egg viability, and longevity) of *Ae. aegypti*, suggesting that improvements in the delivery of dsRNA that could knock down gene expression might still be a viable strategy to pursue as it does not appear to have significant negative effects on fitness parameters.

Chapter 3.

Antibiotic treatment of *Aedes aegypti* prior to feeding with engineered midgut bacteria to reduce the gene expression of cathepsin B

3.1. Introduction

Bacteria colonize the intestine and midgut of most mosquito species [15]. Midgut bacterial communities in mosquitoes mainly consist of Gram-negative aerobes and facultative anaerobes [145]. In females, midguts are primarily colonized by members of the *Gammaproteobacteria*, which are common in hematophagous insects [15]. Interestingly, *Pseudomonas spp*, *Serratia spp.*, and *Enterobacter spp.* are also frequently found in females. As for males, their midguts are dominated by bacteria from the phylum Firmicutes such as those of the genera *Staphylococcus*, *Bacillus*, *Paenibacillus,* and *Micrococcus*.

Female acquire most of their bacteria through feeding [15]. At the larval stage, mosquitoes consume bacteria and plankton from their aquatic habitat. However, only a small portion of these microbiota are retained through metamorphosis. Bacterial diversity in *Ae. aegypti* declined from 74 operational taxonomic units (OTUs) in larvae to 39 OTUs and 22 OTUs in adult females before and after blood-feeding [41]. There is no doubt that bacteria remain in the GI tract as they move through different larval stages, but how many survive metamorphosis is less clear, especially as females imbibe water immediately upon emergence, and may replenish bacteria removed during metamorphosis [41].

Adult mosquitoes also acquire new microflora from plants as they forage for sucrose and females may acquire microbes from the skin of vertebrates as they probe prior to blood-feeding. Some bacteria may be transmitted transovarially, for example, the symbiont *Wolbachia* sp. is transmitted via vertical transmission in mosquitoes [15], and venereal transmission of bacteria such as *Asaia* is also possible in *An. gambiae* and *An. stephensi* [146].

Bacteria may contribute to the well-being of mosquitoes. Larvae of *An. stephensi* that were treated with antibiotics had delayed larval development and an asynchrony in the appearance of later instars [147]. However, if the antibiotic treatment was accompanied by the feeding of an antibiotic-resistant mutant strain of *Asaia*, larval development was normal. The antibiotic-induced effects on larval development could be rescued by bacteria from the aquatic habitat of conventionally reared larvae, such as *Escherichia coli* [41].

Midgut bacteria in *Ae. aegypti* are involved in the digestion of a bloodmeal, which is associated with egg production. These midgut bacteria contribute to mosquito digestion by producing lytic enzymes which facilitate the assimilation of complex molecules [148]. The midgut microbiome also may affect vector competence [141].

Microbial interactions that occur between pathogens and microbiota are complex and may affect mosquito traits such as vector competence. These interactions may be direct or indirect, cooperation or competition [15]. *Wolbachia* sp. can interfere with replication and dissemination of pathogens such as dengue and chikungunya in *Ae. aegypti* [83–85]. In addition, the introduction of *Proteus* sp. and *Paenibacillus* sp. into *Ae. aegypti* after antibiotic treatment to eliminate resident microbes significantly reduced the susceptibility of *Ae. aegypti* to DENV infection [149,150].

The previous chapter described efforts to reduce the expression of cathepsin B in mosquitoes using a bacterial expression system. The failure to reduce cathepsin B may be due to the dilution of our bacteria by resident microflora. Other studies have treated mosquitoes with antibiotics to eliminate bacteria and then replace them with a specific bacterium of interest [149,150]. In this chapter, we used this strategy to treat *Ae. aegypti* females with antibiotics to eliminate resident bacteria, and then allow them to ingest the bacteria engineered to express the dsRNA to knockdown the expression of cathepsin B (HT115-CAT) or a plant gene (HT115-ANT). These experiments are designed to determine if our mixed results in Chapter 2 could be rescued by prior antibiotic treatment.

3.2. Methods

3.2.1. Mosquito rearing

Our laboratory colony of *Ae. aegypti* (Liverpool strain) was maintained under standard laboratory conditions at 27 ± 2℃, 70% relative humidity and a 12:12 hours light:dark cycle. Eggs were laid on wet filter papers and hatched in autoclaved water. Larvae developed in water trays containing distilled water and were fed with crushed fish food. Adults were kept in cardboard cages with netting on top.

3.2.2. Antibiotic treatment

Newly emerged females were fed with (i) 100 µg/ml of ampicillin (Sigma-Aldrich, Saint Louis MO) for 1 day (ii) 20 U (µg)/ml of penicillin-streptomycin (ThermoFisher Scientific, Waltham MA) in 10% sucrose solution for multiple days, or (iii) an antibiotic cocktail of 75 µg/ml of gentamicin sulfate (Life Technologies, Carlsbad CA) and 100 U (µg)/ml of penicillin-streptomycin (ThermoFisher Scientific, Waltham MA) in 10% sucrose solution for 3 days [149,151]. Controls were fed with sterile 10% sucrose solution. A drop of sterile blue food dye was added to the antibiotic-sucrose solution for visual confirmation that the insects had ingested the solution [149]. Feeding was done *ad libitum* by placing sterile cotton balls moistened with antibiotic-sucrose or sucrose only solution on mosquito cages. After treatment, mosquitoes were anesthetized using carbon dioxide (CO_2), examined under a microscope for blue abdomens, and transferred to new cages for subsequent colony forming unit (CFU) assays.

3.2.3. Colony forming unit (CFU) assay

CFU assays were performed at different times after antibiotic ingestion under sterile conditions. All tools used in these experiments were autoclaved and wiped with 70% ethanol. Ampicillin treated mosquitoes were anesthetized with CO_2, and surface sterilized by submerging them into 70% ethanol for 5 mins, followed by washing with PBS twice. Mosquitoes were left in PBS until midgut dissections were performed. After treatment with ethanol, penicillin-streptomycin treated or gentamicin sulfate, penicillin-streptomycin treated mosquitoes were transferred into a tube containing autoclaved water and vortexed briefly to remove the ethanol, were vortexed in 10% bleach for 1 min,

and washed twice in autoclaved water. After surface sterilization, mosquitoes were dried on autoclaved filter paper. Midguts were dissected from the adults in a drop of sterile PBS and homogenized in 100 µl of sterile LB broth for 1 min. Contamination controls involved placing a surface sterilized mosquito in PBS on a microscope slide, placing the dissecting needle in the PBS and subsequently placing the dissecting needle into a tube that contained sterile LB media. These tubes were treated identically as the tubes containing samples. All sample or control homogenates were aseptically plated onto LB plates and assessed for bacterial growth after incubation at 37℃ for 1 day. For gentamicin sulfate, penicillin-streptomycin treated mosquitoes, midgut dissections were not performed as described. Instead, after ethanol surface sterilization, each entire mosquito was homogenized in 100 µl of LB media for 1 min and plated on LB plates [150].

3.2.4. Preparation of bacteria for feeding

The bacteria used in these studies and the cloning and verification of the constructs were described in Chapter 2. HT115-CAT and HT115-ANT were grown in LB media containing 50 µg/ml of ampicillin and 12.5 µg/ml of tetracycline overnight at 37℃, 200 rpm. Overnight cultures were diluted to OD_{600} of ~0.1 in 2X YT media containing antibiotics, grown to OD_{600} of 0.4 – 0.6, and induced with IPTG to a final concentration of 2 mM. IPTG was not added to the uninduced control. After 4 hours of induction at 37℃ at 200 rpm, bacteria were harvested by centrifugation at 4,400 x g for 10 mins at 4℃ and resuspended with 10% sterile sucrose to a concentration of 1×10^{10}cfu/ml for feeding.

For the subsequent 3 days after feeding on the antibiotic cocktail (75 µg/ml of gentamicin sulfate (Life Technologies, Carlsbad CA) and 100 U (µg)/ml of penicillin-streptomycin (ThermoFisher Scientific, Waltham MA)) in 10% sucrose solution for 3 days, 50 mosquitoes were fed a 10% sucrose solution containing bacteria with the constructs HT115-CAT, HT115-ANT, uninduced HT115-CAT, or no bacteria controls.

In order to determine the levels of expression of cathepsin B, after antibiotic treatment, mosquitoes were surface sterilized with 70% ethanol, and then rinsed twice with sterile water. The midguts of mosquitoes were then dissected in a drop of sterile PBS. At each time point, 7 midguts were pooled for total RNA extraction using 200 µl of TRI Reagent following manufacturer's protocols. The extracted RNA was resuspended in DEPC water, quantified using a NanoDrop 2000C (ThermoFisher Scientific, Waltham

MA), and stored at – 80℃. RNaseOFF Ribonuclease Inhibitor (Applied Biological Materials, Richmond BC) was added to each RNA sample to a final concentration of 1 U/µl before DNase treatment was performed using TURBO DNA-free Kit (ThermoFisher Scientific, Waltham MA). RNA integrity and DNA contamination were evaluated as described in Chapter 2. Subsequently, 400 ng of total RNA was reverse transcribed in 20 µl cDNA synthesis reactions containing 4µl of 5X All-In-One RT MasterMix as follows: 65℃ for 5 mins, 42℃ for 50 mins and 70℃ for 15 mins. Each cDNA was used in a standard PCR to amplify a constitutively expressed housekeeping gene, β-actin (LOC5574526) (see Appendix A for primer sequences). The PCR was done in 25 µl reactions containing 12 µl Safe-Green 2X PCR Taq MasterMix ,1 µl of each primer (10 µM) and 1 µl of cDNA, with the following settings: 95℃ for 2 mins, 40 cycles of 95℃ for 10 secs, 55℃ for 10 secs and 72℃ for 30 secs.

Subsequently, cathepsin B expression was measured using qPCR. One µl of cDNA was used in 12 µl qPCR reactions that contained 6 µl of PerfeCTa SYBR Green SuperMix, 1 µl of each primer (10 µM) and 1 µl of cDNA that was diluted to 15 ng/µl with NFH_2O (see qPCR-cathepsin B primer sequences in Appendix A). A no template control was also included. All samples were run in duplicate using the settings: 95℃ for 2 mins, followed by 40 cycles of 95℃ for 10 secs, 50℃ for 15 secs and 72℃ for 20 secs. β-actin was used as a reference housekeeping gene for normalization (see qPCR- β-actin primer sequences in Appendix A). Data were analyzed using the $2^{\Delta CT}$ and $2^{-\Delta\Delta CT}$ to calculate relative expression levels [137]. The change in gene expression was considered statistically significant when a 2-fold change was achieved. Melt curve analysis was done to check for nonspecific amplification.

3.2.5. Statistical analysis

The impact of antibiotic treatment on the elimination of midgut microbiota in *Ae. aegypti* were assessed using generalized linear model fit analysis with a binomial distribution and a logit link via JMP statistical software 13.1.0 (SAS Institute).

3.3. Results

3.3.1. Antibiotic treatment

Ampicillin treatment did not eliminate the midgut microbiota of *Ae. aegypti* (Table 3.1). There was no significant difference between antibiotic-treated and untreated mosquitoes (generalized linear model fit analysis with a binomial distribution and a logit link, DF = 1, ChiSquare = 0.08, P-value = 0.78). The contamination controls had no bacteria growth. Details about the analysis were shown in Appendix F.

Table 3.1 Presence of bacteria in midguts of *Aedes aegypti* females that were fed with 100 µg/ml of ampicillin in 10% sucrose for 1 day. No antibiotic controls were fed with 10% sucrose solution. The proportion of mosquitoes with bacteria represents the mean of 2 repeats, n = 5 per repeat.

condition	the proportion of mosquitoes with bacteria	lower 95% mean	upper 95% mean
antibiotic	0.80	0.11	0.99
no antibiotic	0.90	0.08	1.00

Penicillin-streptomycin treatment failed to eliminate bacteria in the midguts of mosquitoes (Table 3.2). We could not generate aseptic mosquitoes, even after we increased the duration of antibiotic treatment to 6 days. The contamination controls had no bacteria growth.

Table 3.2 Presence of bacteria in midguts of *Aedes aegypti* females that were fed with 20 U (µg)/ml of penicillin-streptomycin in 10% sucrose for multiple days. No antibiotic controls were fed with 10% sucrose solution. No repeat, n = 5.

number of antibiotic treatment days	the proportion of mosquitoes with bacteria	
	antibiotic	no antibiotic
1	0.80	1.00
3	0.60	1.00
4	0.60	1.00
5	0.80	1.00
6	1.00	1.00

Mosquitoes that received a cocktail of gentamicin sulfate, penicillin and streptomycin for 3 days had a significantly lower proportion of mosquitoes with bacteria

than no antibiotic controls on all 3 days (generalized linear model fit analysis with a binomial distribution and a logit link, DF = 1, ChiSquare = 4.63, 4.73, 4.27, P-value = 0.03, 0.03, 0.04 for day 1, 2, 3 respectively). The contamination controls had no bacteria growth. Details about the analysis were shown in Appendix G.

Table 3.3 Presence of bacteria in *Aedes aegypti* females that were fed with 75 µg/ml of gentamicin sulfate and 100 U (µg)/ml of penicillin-streptomycin in 10% sucrose for 3 days. No antibiotic controls were fed with 10% sucrose solution. After antibiotic treatment, mosquitoes were fed with 10% sucrose and CFU assays were performed for 3 days. The proportion of mosquitoes with bacteria represents the mean of 3 repeats, n = 10 per repeat.

day	condition	the proportion of mosquitoes with bacteria	lower 95% mean	upper 95% mean
1	antibiotic	0.17	0.01	0.80
	no antibiotic	0.97	0.05	1.00
2	antibiotic	0.23	0.02	0.82
	no antibiotic	1.00	0.00	1.00
3	antibiotic	0.20	0.01	0.80
	no antibiotic	0.97	0.05	1.00

3.3.2. Assessment of cathepsin B expression after antibiotic treatment

The expression of cathepsin B in mosquitoes that received the HT115-CAT construct was significantly reduced compared with the no bacteria control at day 1 and 3, suggesting that the engineered bacteria were in fact resulting in an overall reduction in cathepsin B expression (Figure 3.1). These data were confounded, however by the fact that a similar pattern of expression occurred when the mosquitoes fed on the HT115-ANT construct or no bacteria (Figure 3.2). In this case, there was also a reduction in cathepsin B expression in the mosquitoes that ingested bacteria expressing dsRNA, even though it did not target a mosquito gene.

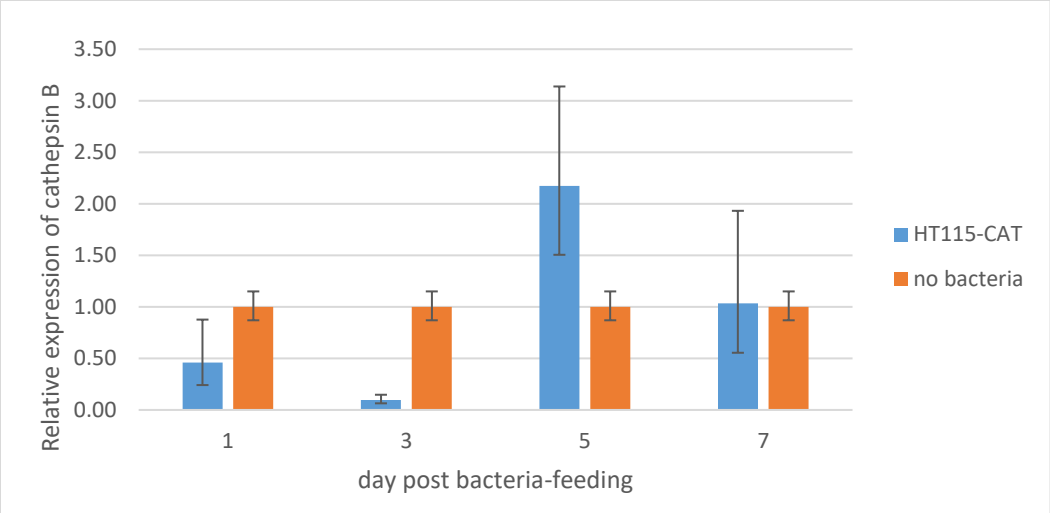

Figure 3.1 **Expression of Cathepsin B in the midguts of *Aedes aegypti* females that received an antibiotic cocktail for three days before ingesting HT115-CAT (blue bars) or a no bacteria control (orange bars). Expression levels in the no bacteria controls were arbitrarily set at 1 and expression levels in the induced HT115-CAT bars represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

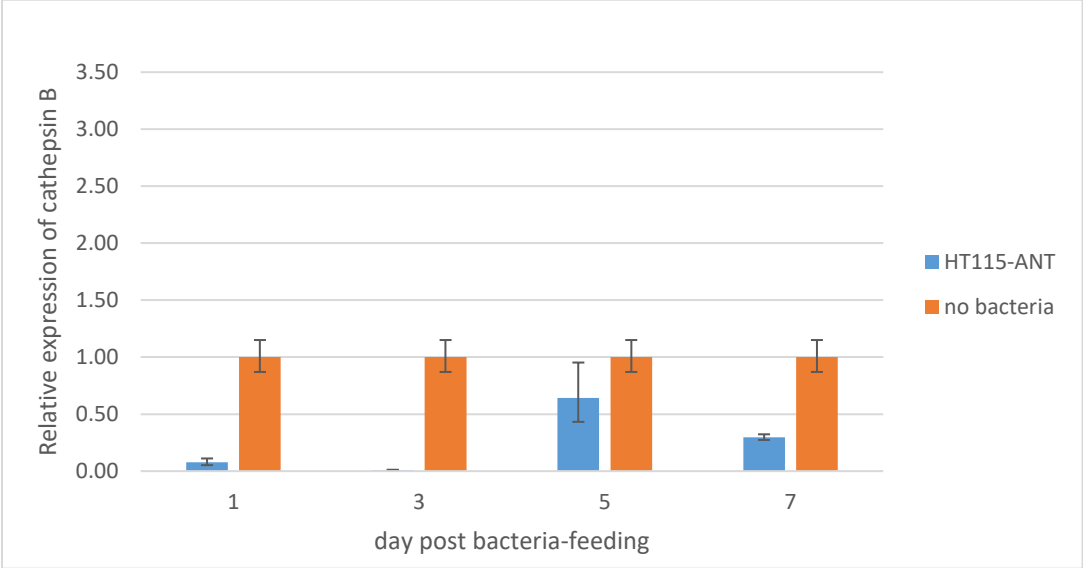

Figure 3.2 **Expression of cathepsin B in the midguts of *Aedes aegypti* females that received an antibiotic cocktail for three days before ingesting HT115-ANT (blue bars) or a no bacteria control (orange bars). Expression levels in the no bacteria controls were arbitrarily set at 1 and expression levels in the induced HT115-ANT bars represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

When we compared the expression in groups of mosquitoes, whether they received the antibiotic cocktail or not, and then both groups were fed with HT115-CAT to knock down cathepsin B expression, we observed a reduction in cathepsin B expression in mosquitoes that did not ingest the antibiotic cocktail (Figure 3.3). Those that ingested the antibiotics had a higher expression. We would expect that replacing the resident bacteria with HT115-CAT would reduce cathepsin B expression.

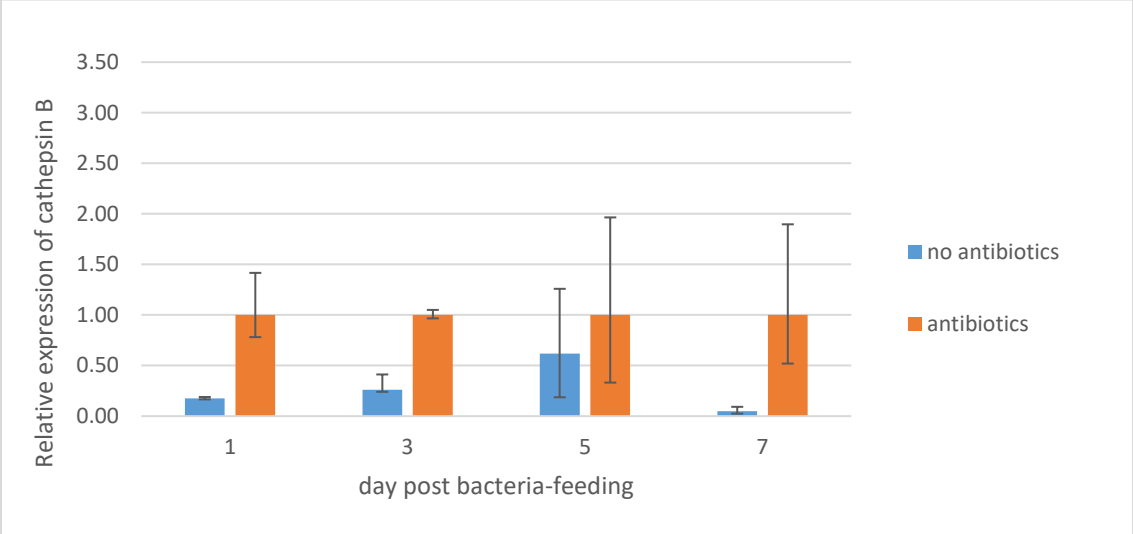

Figure 3.3 **Expression of cathepsin B in the midguts of *Aedes aegypti* females that received (orange bars), or did not receive (blue bars), an antibiotic cocktail for three days before ingesting HT115-CAT. Expression levels in the antibiotics+ group were arbitrarily set at 1 and expression levels in the group that did not receive antibiotics represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

When mosquitoes were fed with the antibiotic cocktail in sucrose solution or only with sucrose solution, and then were fed with the HT115-ANT construct that expressed dsRNA corresponding to a plant gene, there was more expression in the group that received no antibiotics at day 3.

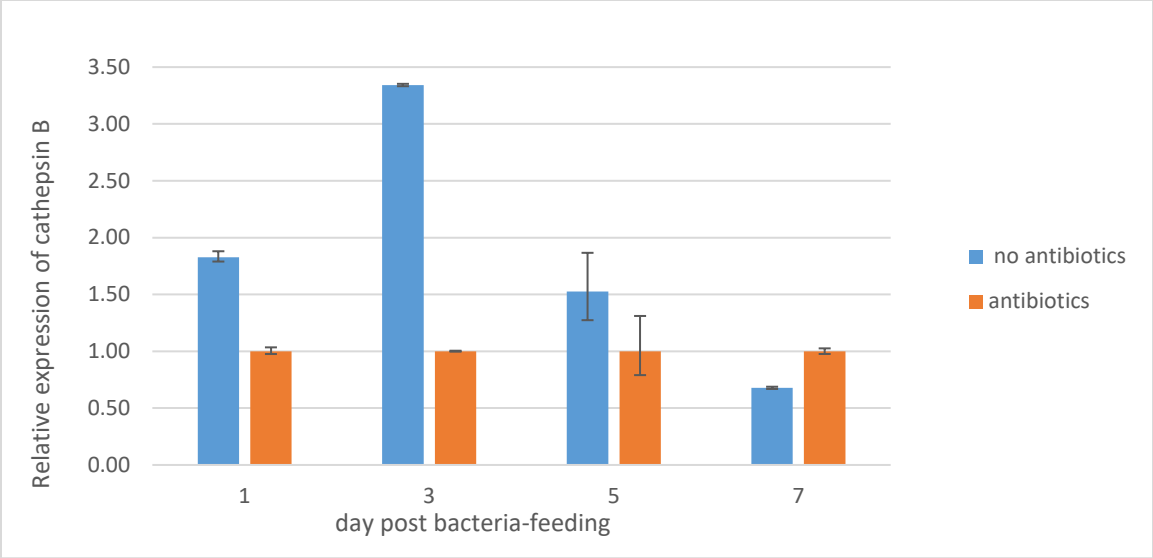

Figure 3.4 **Expression of cathepsin B in the midguts of *Aedes aegypti* females that received, or did not receive an antibiotic cocktail for three days before ingesting bacteria engineered to knock down the expression of a plant gene. Expression levels in the antibiotics + group were arbitrarily set at 1 and expression levels in the group that received no antibiotics represent relative fold-differences in expression. Error bar represents the standard deviation of technical duplicates, n = 7.**

3.4. Discussion

Bacteria were detected in more than half of the ampicillin-treated and penicillin-streptomycin-treated mosquitoes. Ampicillin resistant bacteria such as *Elizabethkingia meningoseptica* and *Serratia marcescens* have been reported from *Ae. aegypti* [152]. A reduction in the proportion of mosquitoes with positive results occurred with the 2 and then 3 antibiotic cocktails, as might be expected. With gentamicin sulfate, penicillin-streptomycin cocktails, we could eliminate microbiota in a significantly larger proportion of mosquitoes. We could also keep these antibiotic-treated mosquitoes aseptic for 3 days under sterile conditions.

The contamination and LB controls provided good indications that CFU assays could be done aseptically without introducing false positive results. We assume that the bacteria found in the CFU assays came from the mosquito midguts instead of the surface of the mosquitoes or the environment. The feeding indicator also helped us ensure that the CFU assay results were not false positives due to the lack of feeding on

antibiotics by mosquitoes. It demonstrated that mosquitoes were septic even after antibiotic treatment.

Interestingly, some of the no antibiotic controls were aseptic. These data have no simple explanation as all larvae were hatched together, lived together, pupated together, and were combined in cartons as adults together, and we would expect similar midgut microbiota [153].

There may be a time factor involved in the comparative studies as other reports expose insects to antibiotics for different time periods, 1-3 days [142, 145]. However, our data in Table 3.2 suggest that treating mosquitoes with antibiotics for multiple days did not improve the elimination of mosquito midgut microbiota.

We treated the mosquitoes with antibiotics and then fed them with bacteria engineered to express dsRNA to knock down cathepsin B expression (HT115-CAT), or dsRNA that codes for a plant gene (HT115-ANT). While the feeding of HT115-CAT did knock down expression of cathepsin B at days 1 and 3. This result becomes confusing, however, when the same lack of cathepsin B expression occurs in mosquitoes that received HT115-ANT carrying the construct to knock down a plant gene. Similarly, when we knocked down the bacterial population by treating with antibiotics, and then fed insects with the HT115-CAT bacteria, we would expect to see a reduction in cathepsin B expression compared with those that had not received antibiotics. Instead, the reverse occurred. And in the case where the insects received HT115-ANT after antibiotic treatment, the data were unclear; those that received no antibiotics had a higher level of cathepsin B expression at day 3.

We questioned whether the timing of removing antibiotics and exposure to HT115 constructs may have had a residual effect on the microbiome. A previous study showed that when introducing different concentrations of *E. coli* into mosquitoes after antibiotic treatment with gentamicin sulfate and penicillin-streptomycin, mosquitoes turned septic from day 1 even at the lowest concentration of fed bacteria [154]. This suggests that the effects we saw should not be a result of carryover antibiotics affecting the HT115 bacteria.

We modified the protocol by homogenizing the entire mosquitoes in LB media, instead of only the mosquito midguts as has been described elsewhere [150]. The entire

mosquito homogenization method had several advantages over the midgut homogenization method. Firstly, the entire mosquito homogenization method was less labour-intensive and time-consuming than the midgut homogenization method, because the midgut dissection step was omitted. Secondly, the simplicity of this method could minimize contamination that could potentially be introduced when performing dissections. Thirdly, the midgut homogenization method could only provide information about the mosquito midgut microbiota. By using the entire mosquito homogenization method, we demonstrated that antibiotic treatment had a systemic effect on each mosquito because the microbiota of the entire mosquito was eliminated after treatment. This finding would be helpful if we were to conduct research on microbiota from other body parts of the mosquitoes. However, the lack of specificity in this method had its drawbacks. By using the entire mosquito homogenization method, we were unsure whether bacteria were from midguts or other parts of the mosquitoes when the CFU assays resulted in bacterial growth.

3.5. Conclusion

We successfully eliminated the microbiome from most of our mosquitoes by feeding them with an antibiotic cocktail of 75 µg/ml of gentamicin sulfate and 100 U (µg)/ml of penicillin-streptomycin in 10% sucrose solution for 3 days. The treated groups had a significantly lower proportion of septic mosquitoes than no antibiotic controls. Antibiotic-treated mosquitoes could be kept aseptic for 3 days under sterile conditions. By using the entire mosquito homogenization method, we determined that antibiotic treatment had a systemic effect on each mosquito.

However, we expected that by reducing the microbiome in the midguts of the insects and replacing the bacteria with HT115-CAT that we would increase the proportion of bacteria with this construct and that this would result in a reduction in cathepsin B expression. We were not able to reduce cathepsin B expression in any logical or predictable fashion.

Chapter 4.

Summary

In Chapter 2, we generated *E. coli* HT115 (DE3) strains that could express dsRNA to target knockdown the expression of cathepsin B or aintegumenta. Although our mosquitoes fed on a high concentration of bacteria in sucrose solution (1×10^{10} CFU/ml), the knockdown of cathepsin B via bacteria-delivered dsRNA was not as effective as microinjection. Our qPCR results show that we could not obtain consistent knockdown of cathepsin B expression using bacteria-delivered dsRNA of cathepsin B.

The ingestion of large amounts of bacteria, whether they expressed dsRNA or not, did not significantly affect adult fecundity or longevity, nor the proportion of eggs that hatched. However, mosquitoes that blood-fed and oviposited had significantly lower lifespans than mosquitoes that did not blood-fed. This suggests that reproduction had a fitness cost.

In Chapter 3, we developed an antibiotic treatment protocol to eliminate or reduce midgut microbiota. Feeding mosquitoes with 100 µg/ml of ampicillin or 20 U (µg)/ml of penicillin-streptomycin in 10% sucrose solution did not eliminate mosquito midgut microbiota. We eliminated microbiota from a significantly large proportion of our mosquitoes by feeding them with 75 µg/ml of gentamicin sulfate and 100 U (µg)/ml of penicillin-streptomycin in 10% sucrose solution for 3 days. The antibiotic treatment had a systemic effect on mosquitoes. Our findings would be useful for future experiments that involved mosquitoes and bacteria.

Treating mosquitoes with gentamicin sulfate, penicillin, and streptomycin prior to bacteria-feeding reduced the expression of cathepsin B as expected. Antibiotic-treated mosquitoes fed with HT115-ANT, however, also had reduced cathepsin B expression.

Mosquitoes fed with HT115-CAT that received antibiotic treatment had higher cathepsin B expression than those that did not receive antibiotic treatment prior to bacteria-feeding, which was unexpected. The reverse occurred in mosquitoes fed with HT115-ANT. Thus, our qPCR results were unpredictable.

Mosquitoes serve as vectors for many parasites and pathogens. We know that the parasites utilize host molecules and resources to survive. Injecting dsRNA to reduce the expression of cathepsin B reduced overall expression, and altered the phenotype from the dengue-susceptible to the dengue-refractory phenotype [18]. We copied a protocol that used bacteria to express dsRNA in *R. prolixus* to knockdown gene expression [119]. However, we could not replicate the success demonstrated in their paper. Originally, we thought that we could establish sugar feeding stations in arid areas to which mosquitoes would be attracted, and where we could introduce to them, bacteria expressing dsRNA to target specific molecules that the pathogens require to survive. Based on the data generated here we are not yet ready for such an approach.

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Appendix A.

Primer sequences

Gene	Primer sequence (5' - 3')
cathepsin B	F:CCGGTGCTCAATATCCAAGT R:ACCCTTCTCGACCCAGAACT
cathepsin B + T7 promoter	F:TAATACGACTCACTATAGGGCCGGTGCTCAATATCCAAGT R:TAATACGACTCACTATAGGGACCCTTCTCGACCCAGAACT
aintegumenta	F:GGTGGAGGATTTCTTTGGGACC R:ACGCCTCGGTATTGAGAAGTTCG
aintegumenta +T7 promoter	F:GTAATACGACTCACTATAGGGCGAATTGGGTGGAGGATTTCTTTGGGACC R:GTAATACGACTCACTATAGGGCGAATTGACGCCTCGGTATTGAGAAGTTCG
actin-5	F:CGTTCGTGACATCAAGGAAA R:GAACGATGGCTGGAAGAGAG
qPCR-cathepsin B	F:CAGGGATGTCACCCATATC R:GAAAGCAGCCTGAACTGG
qPCR- β- actin	F:AAGGCTAACCGTGAGAAGATGAC R:GATTGGGACAGTGTGGGAGAC

Appendix B.

Supplementary data for Chapter 2

Table B.1 **Sequencing results of cathepsin B (CAT) and aintegumenta (ANT). T7 promoters are shown in blue.**

Gene	Sequence
CAT	TAATACGACTCACTATAGGGGNNNNNNNNNNNCGNTNNGNATCCCACAAGTTGCATTTCTC CCTCGTTTGAGGGTGCACANCACAAATAACCCCCGTGGAGATGTTCCGTTTCGTGTTGGTA CTGGCCTGCTGCCAGGCCGCATTGGCCCAAGGCTACCGCACTCCGTCCCCTAAGTTCTCAT CNAAAAACTCTGCCCGGTACCCAGACAATCAGTACAACGAATACTTGGGTGAATATTCCGGT GCTCAATATCCAAGTCCCTATAGTGAGTCGTATTA
ANT	GTAATACGACTCACTATAGGGCGAATTGGGAGAGTANNCAGTGAACCACAACCAGTCCTAN NTTNNAGCCAGATTCNNNAACACCGACNTGTTGGCATCCNANTTCANTCACGTGACNAGAA CATGNANAGCCGGCAGCAACCCGAAAACTCCNGCAGGATACNNATNNGGTGTNGACCTGG CTNACCTGGAGNGCANTANNCTGCCGCTGGNANTCANGNNTGANNTCNAAAATTTNNANNT TGCGGAGANNTTCNANNNNTGTGANCAATTCGCCCTATAGTGAGTCGTATTAC

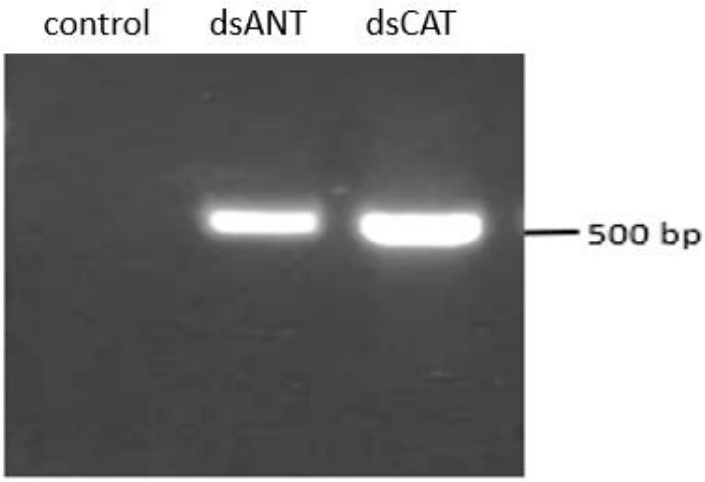

Figure B.1 **Double-stranded RNA extraction from HT115-CAT and HT115-ANT.**

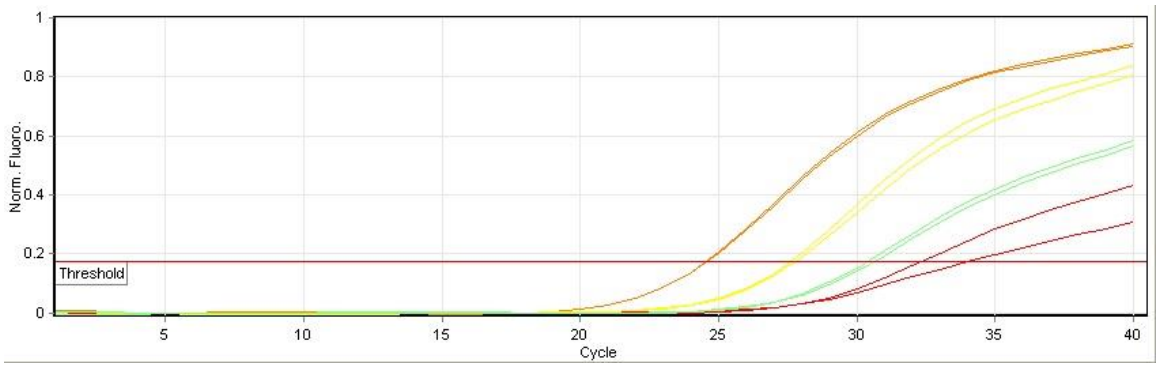

Figure B.2 **Serial dilutions of cathepsin B qPCR amplification curve. Dilutions: 1X (red), 1/10X (yellow), and 1/100X (green). The purple lines represent the negative control.**

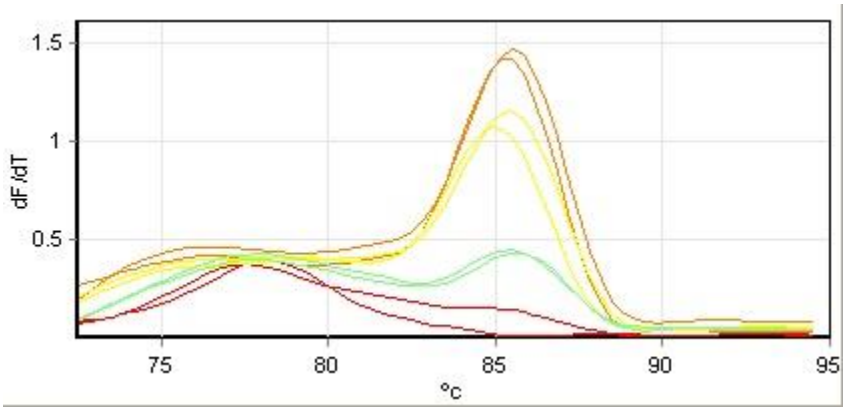

Figure B.3 **Serial dilutions of cathepsin B qPCR melt curve. Dilutions: 1X (red), 1/10X (yellow), and 1/100X (green). The purple lines represent the negative control.**

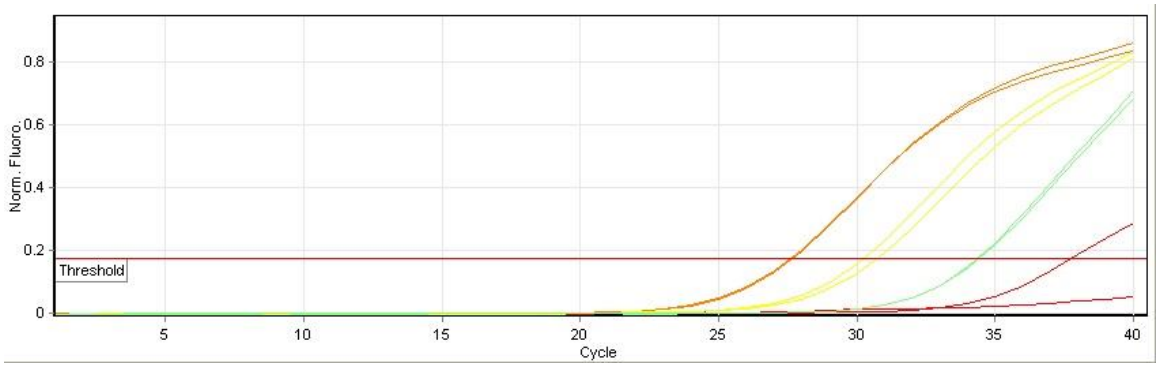

Figure B.4 **Serial dilutions of β-actin qPCR amplification curve. Dilutions: 1X (red), 1/10X (yellow), and 1/100X (green). The purple lines represent the negative control.**

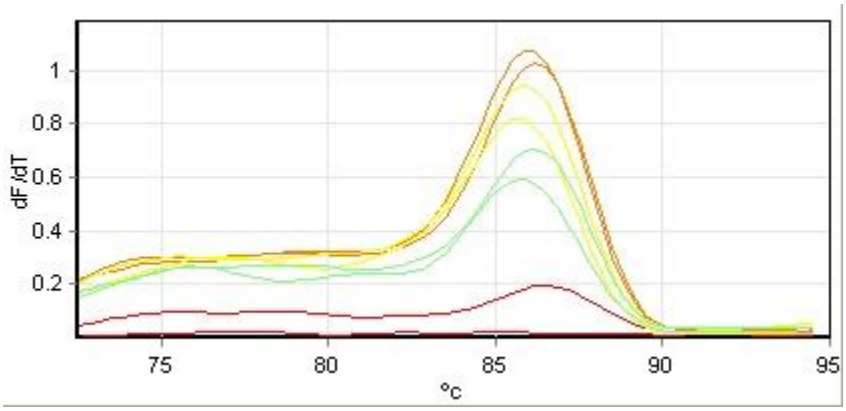

Figure B.5 **Serial dilutions of β-actin qPCR melt curve. Dilutions: 1X (red), 1/10X (yellow), and 1/100X (green). The purple lines represent the negative control.**

Appendix C.

Least square means analysis for fecundity test

Table C.1 Summary of Fit

RSquare	0.62
RSquare Adj	0.49
Root Mean Square Error	10.51
Mean of Response	76.64
Observations (or Sum Wgts)	9

Table C.2 REML Variance Component Estimates

Random Effect	Var Ratio	Var Component	Std Error	95% Lower	95% Upper	Wald p-Value	Pct of Total
Repeats	0.8450627	93.305871	132.68738	-166.7566	353.36835	0.4819	45.801
Residual		110.41295	78.073748	39.633891	911.7153		54.199
Total		203.71882	140.13485	74.654105	1545.97		100.000

Note: Total is the sum of the positive variance components.

-2 LogLikelihood = 53.27

Total including negative estimates = 203.72

Table C.3 Fixed Effect Tests

Source	Nparm	DF	DFDen	F Ratio	Prob > F
condition	2	2	4	0.40	0.69

Appendix D.

Generalized linear model fit analysis for egg viability test

Overdispersion parameter estimated by Pearson Chisq/DF

Response: Mean proportion of egg hatched

Distribution: Binomial

Link: Logit

Estimation Method: Firth Adjusted Maximum Likelihood

Observations (or Sum Wgts) = 9

Table D.1 Whole Model Test

Model	-LogLikelihood	L-R ChiSquare	DF	Prob>ChiSq
Difference	0.04796529	0.0959	2	0.9532
Full	4.39445611			
Reduced	4.44242139			

Goodness Of Fit Statistic	ChiSquare	DF	Prob>ChiSq	Overdispersion
Pearson	3.0691	6	0.8001	1.0000
Deviance	3.5762	6	0.7338	

AICc
26.7889

Table D.2 Effect Tests

Source	DF	L-R ChiSquare	Prob>ChiSq
Condition	2	0.09593	0.9532

Table D.3 Parameter Estimates

Term	Estimate	Std Error	L-R ChiSquare	Prob>ChiSq	Lower CL	Upper CL
Intercept	-0.555609	0.6968888	1.1084248	0.2924	-1.845586	0.5964635
Condition[induced]	0.0507051	0.9793011	0	1.0000	-1.76236	1.7349289
Condition[no bacteria]	0.2334059	0.9704295	0.060747	0.8053	-1.518008	1.9307629

Appendix E.

Least square means analysis for longevity test

Without blood-feeding

Table E.1 Summary of Fit

RSquare	-5.86543
RSquare Adj	-8.1539
Root Mean Square Error	4.392544
Mean of Response	37.94444
Observations (or Sum Wgts)	9

Table E.2 REML Variance Component Estimates

Random Effect	Var Ratio	Var Component	Std Error	95% Lower	95% Upper	Wald p-Value	Pct of Total
Repeats	-0.313389	-6.046667	4.563996	-14.99193	2.8986011	0.1852	0.000
Residual		19.294444	13.643233	6.9259438	159.32044		100.000
Total		19.294444	13.643233	6.9259438	159.32044		100.000

Note: Total is the sum of the positive variance components.

-2 LogLikelihood = 34.646831105

Total including negative estimates = 13.247778

Table E.3 Fixed Effect Tests

Source	Nparm	DF	DFDen	F Ratio	Prob > F
condition	2	2	4	0.6659	0.5628

With blood-feeding and oviposition

Table E.4 Summary of Fit

RSquare	0.678063
RSquare Adj	0.570751
Root Mean Square Error	2.855307
Mean of Response	34.04444
Observations (or Sum Wgts)	9

Table E.5 REML Variance Component Estimates

Random Effect	Var Ratio	Var Component	Std Error	95% Lower	95% Upper	Wald p-Value	Pct of Total
Repeats	0.4650767	3.7916667	6.786981	-9.510572	17.093905	0.5764	31.744
Residual		8.1527778	5.7648845	2.9265253	67.320111		68.256
Total		11.944444	7.5591716	4.6518495	71.967615		100.000

Note: Total is the sum of the positive variance components.

-2 LogLikelihood = 36.857434552

Total including negative estimates = 11.944444

Table E.6 Fixed Effect Tests

Source	Nparm	DF	DFDen	F Ratio	Prob > F
condition	2	2	4	3.1140	0.1529

With and without blood-feeding

Table E.7 Summary of Fit

RSquare	0.359678
RSquare Adj	0.222466
Root Mean Square Error	3.448577
Mean of Response	35.99444
Observations (or Sum Wgts)	18

Table E.8 Parameter Estimates

Term	Estimate	Std Error	DFDen	t Ratio	Prob>|t|
Intercept	35.994444	0.579058	2	62.16	0.0003*
condition[induced]	-1.811111	1.149526	12	-1.58	0.1411
condition[no bacteria]	2.8055556	1.149526	12	2.44	0.0311*
bloodfeeding[no]	1.95	0.812837	12	2.40	0.0336*

Table E.9 REML Variance Component Estimates

Random Effect	Var Ratio	Var Component	Std Error	95% Lower	95% Upper	Wald p-Value	Pct of Total
Repeats	-0.082083	-0.976188	1.2910008	-3.506503	1.5541268	0.4496	0.000
Residual		11.892685	4.8551684	6.1153651	32.406693		100.000
Total		11.892685	4.8551684	6.1153651	32.406693		100.000

Note: Total is the sum of the positive variance components.

-2 LogLikelihood = 83.499571734

Total including negative estimates = 10.916497

Table E.10 Fixed Effect Tests

Source	Nparm	DF	DFDen	F Ratio	Prob > F
condition	2	2	12	3.0624	0.0842
bloodfeeding	1	1	12	5.7552	0.0336*

Appendix F.

Generalized linear model fit analysis for ampicillin test

Response: Mean proportion of mosquitoes with bacteria

Distribution: Binomial

Link: Logit

Estimation Method: Maximum Likelihood

Observations (or Sum Wgts) = 4

Table F.1 Whole Model Test

Model	-LogLikelihood	L-R ChiSquare	DF	Prob>ChiSq
Difference	0.03986556	0.0797	1	0.7777
Full	1.26230815			
Reduced	1.30217371			

Goodness Of Fit Statistic	ChiSquare	DF	Prob>ChiSq
Pearson	0.7222	2	0.6969
Deviance	0.9551	2	0.6203

AICc
18.5246

Table F.2 Effect Tests

Source	DF	L-R ChiSquare	Prob>ChiSq
condition	1	0.0797311	0.7777

Table F.3 Parameter Estimates

Term	Estimate	Std Error	L-R ChiSquare	Prob>ChiSq	Lower CL	Upper CL
Intercept	1.7917595	1.4731391	2.2031689	0.1377	-0.498249	7.3038153
condition[antibiotic]	-0.405465	1.4731391	0.0797311	0.7777	-5.874912	2.9344471

Appendix G.

Generalized linear model fit analysis for gentamicin sulfate, penicillin-streptomycin treatment

Day 1

Response: Mean proportion of mosquitoes with bacteria

Distribution: Binomial

Link: Logit

Estimation Method: Maximum Likelihood

Observations (or Sum Wgts) = 6

Table G.1 Whole Model Test

Model	-LogLikelihood	L-R ChiSquare	DF	Prob>ChiSq
Difference	2.31527273	4.6305	1	0.0314*
Full	1.2994885			
Reduced	3.61476123			

Goodness Of Fit Statistic	ChiSquare	DF	Prob>ChiSq
Pearson	0.2549	4	0.9925
Deviance	0.2783	4	0.9912

AICc
10.5990

Table G.2 Effect Tests

Source	DF	L-R ChiSquare	Prob>ChiSq
condition	1	4.6305455	0.0314*

Table G.3 Parameter Estimates

Term	Estimate	Std Error	L-R ChiSquare	Prob>ChiSq
Intercept	0.878929	1.7849949	0.32076	0.5712
condition[antibiotic]	-2.488367	1.7849949	4.6305455	0.0314*

Day 2

Response: Mean proportion of mosquitoes with bacteria

Distribution: Binomial

Link: Logit

Estimation Method: Maximum Likelihood

Observations (or Sum Wgts) = 6

Table G.4 Whole Model Test

Model	-LogLikelihood	L-R ChiSquare	DF	Prob>ChiSq
Difference	2.36421604	4.7284	1	0.0297*
Full	1.13299663			
Reduced	3.49721267			

Goodness Of Fit Statistic	ChiSquare	DF	Prob>ChiSq
Pearson	0.1491	4	0.9974
Deviance	0.1660	4	0.9967

AICc
10.2660

Table G.5 Effect Tests

Source	DF	L-R ChiSquare	Prob>ChiSq
condition	1	4.7284321	0.0297*

Table G.6 Parameter Estimates

Term	Estimate	Std Error	L-R ChiSquare	Prob>ChiSq
Intercept	9.0462263	4440.6901	1.0631295	0.3025
condition[antibiotic]	-10.23581	4440.6901	4.7284321	0.0297*

Day 3

Response: Mean proportion of mosquitoes with bacteria

Distribution: Binomial

Link: Logit

Estimation Method: Maximum Likelihood

Observations (or Sum Wgts) = 6

Table G.7 Whole Model Test

Model	-LogLikelihood	L-R ChiSquare	DF	Prob>ChiSq
Difference	2.13551809	4.2710	1	0.0388*
Full	1.4014876			
Reduced	3.53700569			

Goodness Of Fit Statistic	ChiSquare	DF	Prob>ChiSq
Pearson	0.3319	4	0.9877
Deviance	0.3564	4	0.9859

AICc
10.8030

Table G.8 Effect Tests

Source	DF	L-R ChiSquare	Prob>ChiSq
condition	1	4.2710362	0.0388*

Table G.9 Parameter Estimates

Term	Estimate	Std Error	L-R ChiSquare	Prob>ChiSq
Intercept	0.9905007	1.7626798	0.4434832	0.5054
condition[antibiotic]	-2.376795	1.7626798	4.2710362	0.0388*