Comparative Analysis of the Gut Microbiome in Wild and Lab Strains of Anopheles Quadrimaculatus Say, and its Effect on Innate Immunity

Eleanor Marie Moen

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Comparative analysis of the gut microbiome in wild and lab strains of *Anopheles quadrimaculatus* Say, and its effect on innate immunity

By

Eleanor Marie Moen

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in the College of Agriculture and Life Sciences

Mississippi State, Mississippi

August 2018
Comparative analysis of the gut microbiome in wild and lab strains of *Anopheles quadrimaculatus* Say, and its effect on innate immunity

By

Eleanor Marie Moen

Approved:

________________________________
Jonas King
(Major Professor)

________________________________
Jerome Goddard
(Committee Member)

________________________________
Shien Lu
(Committee Member)

________________________________
Jeff Dean
(Department Head)

________________________________
Ken Willeford
(Graduate Coordinator)

________________________________
George Hopper
Dean
College of Agriculture and Life Sciences
Name: Eleanor Marie Moen
Date of Degree: August 10, 2018
Institution: Mississippi State University
Major Field: Agricultural Life Sciences (Entomology)
Committee: Jonas King (Advisor), Jerome Goddard, Shien Lu
Title of Study: Comparative analysis of the gut microbiome in wild and lab strains of *Anopheles quadrimaculatus* Say, and its effect on innate immunity
Pages in Study 47
Candidate for Degree of Master of Science

Vector competence of mosquitoes has been linked to the conditions in which the larvae mature to adults. The microbial community obtained from the rearing environment is suspected to be one key factor in this interplay. A better understanding of how the rearing environment affects the gut microbiome and *Anopheles-Plasmodium* interactions could be useful for understanding observed lab vs. field differences in *Plasmodium* biology and help drive future control efforts. Currently there is a lack of research done on the differences between lab strain mosquitoes and their rearing environments and how lab mosquitoes differ from wild type mosquitoes. Bridging this gap and studying how rearing habitats change gut microbiomes is critical for optimizing the lab-rearing environment. This thesis focuses on the effects larval rearing has on microbiome establishment and innate immune responses in the common malaria mosquito, *Anopheles quadrimaculatus*. 
DEDICATION

I would like to dedicate this research to my friends and family. To my family in Illinois, thank you for your continued support, despite me being so far away from home. Misti and Dad, thank you for always checking in on me and making sure I am doing well. Bridgette, you and Ethan have always pushed me to be better as a person and a scientist, thank you. Ashley, thank you for moving across the country with me to experience a new chapter in my life, your continued support and friendship is truly one of the greatest gifts of my life. And to all of the friends I have made at Mississippi State, thank you for embracing me and inspiring me to do well. Love you all.
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CHAPTER I
INTRODUCTION AND LITERARY REVIEW

Background

Despite efforts made in the past several decades for malaria control, many endemic regions such as Asia, South America, and Sub-Saharan Africa still struggle with the disease. *Plasmodium* is the causative agent of malaria in humans. In the most recent World Malaria Report it showed that, in 2016, there were an estimated 212 million new cases of malaria and 429,000 deaths (WHO 2016). This report is part of a larger analysis looking at the Global Technical Strategy for Malaria (GTSM) from 2012-2030. Included in this report was another analysis that suggested that out of the 91 countries involved in the GTSM, less than half are on track to reduce case incidences to 40% by 2020 (WHO 2016). As outlined in the GTSM, the majority of the prevention efforts for malaria in the areas designated as part of the program are insecticide-treated mosquito nets and indoor residual spraying. While these programs are being implemented in more areas and the rate of residual spraying has increased, *Anopheles* spp. are becoming more resistant to the most commonly used insecticides (WHO 2016, Sokhna *et. al* 2013). Like other mosquito borne diseases, extreme changes in landscape from events such as war, extreme weather, and urban development cause rapid increases in disease prevalence. A more holistic approach to controlling malaria that would help reach the goal for the GTSM, combat the
large diversity of *Plasmodium* spp., and minimize the effects of insecticide resistance in the process is necessary for prolonged control.

**History of malaria and its vectors**

Historically, malaria has been a global problem. Only in relatively recent history the United States and Europe have not had endemic areas. Louis Alphonse Laveran found *Plasmodium* to be the causative agent of malaria in 1880. Ronald Ross made the discovery that the disease vector was the Anopheles mosquito nearly two decades later with the discovery of avian malaria in 1897. It was not until 1951 that the United States reported that malaria had been eradicated by the widespread use of DDT, dichlorodiphenyl-trichloroethane (DDT), and large-scale wetland draining (CDC 2018). Even though eradication of malaria in the United States has been achieved, the primary vector *Anopheles quadrimaculatus* is still present in the eastern United States (Sinka 2013). Since the eradication of human malaria in the United States, there have been 63 reported local transmissions (CDC 2018). A local transmission is when a mosquito bites an infected person that recently traveled, contracted the disease, and then successfully transmitted it to another local mosquito vector (CDC 2018).

The common malaria mosquito, *An. quadrimaculatus*, provides a unique opportunity to study malaria in a mosquito that is considered to not be a threat of spreading a pathogen. In the context of this thesis, this setting provides the opportunity to study a malaria vector native to the southeastern United States that can be reared in a lab. This makes the research relatively less expensive and more holistic. With an arthropod containment level of ACL-1, *An. quadrimaculatus* is a safer mosquito to work with, compared to the most common malaria vector, *An. gambiae*. This is true because *An.
*An. quadrimaculatus* is native to the eastern United States, a region no longer endemic with malaria (CDC 2018). *An. quadrimaculatus* presents a unique opportunity to safely, cost effectively, and holistically study a malaria vector in both wild and laboratory settings.

In the eastern United States, the only vector of malaria is *An. quadrimaculatus* (Sinka 2013, Levine *et al.* 2004). The *An. quadrimaculatus* species complex consists of five sister species that closely resemble one another (Reinert *et al.* 1997). Other global vectors of malaria have similar species complexes, such as *An. gambiae*. This high level of strain dependency makes studying the genetics of a specific mosquito species difficult. Understanding the adult and larval ecological characteristics shared by the sister species is important in vector control and malarial containment.

*An. quadrimaculatus* are reared at a temperature around 74°F and at a relative humidity of 70-80% (Keener 1945). It takes about twenty-one days for a mosquito to progress from oviposition to eclosion (Keener 1945). The majority of this time is spent during the four larval instars (Keener 1945). Females reared in a lab are able to take a blood meal as early as eighteen hours post-emergence (Keener 1945). In a lab-reared female’s lifetime, she can lay up to five broods of eggs (Keener 1945). The preferred oviposition sites are slow moving streams, ponds, and lakes that have aquatic vegetation (Carpenter and LaCasse 1955). Choice of oviposition habitat is important because where the adult female chooses to lay her eggs will be the rearing environment for her offspring. The specific habitat preference of *An. quadrimaculatus* is restricted, but those types of habitats are abundant and can include areas such as flooded rice fields (Marten *et al.* 2000). In the wild, development time is around fourteen days from hatch to emergence and the adults are suspected to live no more than two weeks (CDC 2018). Wild *An.
An. quadrimaculatus are typically active in the late spring and summer months and, if warm enough, in small numbers in the winter (Weidhass et al. 1965).

The larval habitat is important to help develop high fitness and immune responses that later contribute to vector capacity as adults (Figure 1). Maturi et al. (2012) showed that mosquitoes have varying lifespans when reared in different plant types. With the ability to have a longer or shorter life based on rearing environment, there is also the opportunity for more or less blood-feeding events for the mosquito as an adult (Maturi et al. 2012). This increase in blood-feeding events creates a higher risk of contracting a pathogen. Larval habitats, such as swamps for An. quadrimaculatus, are complex. The female’s choice for oviposition site determines the fitness of the mosquito as an adult.

Plasmodium undergoes a two-host cycle between an Anopheles mosquito and a mammal or avian host. Firstly, the vector Anopheles, must bite an infected mammal or bird to ingest Plasmodium gametocytes, the sexual stage of the parasite. After those gametocytes mature to gametes they transform into mobile ookinetes that move to the midgut lining and form oocysts on the midgut’s epithelium. When the oocytes rupture, released sporozoites make their way to the salivary glands. Once the sporozoites reach the salivary glands, the mosquito is infective to vertebrates. The extrinsic incubation period, the interval between vector contracting the causative agent and its ability to transmit the agent, is a week to ten days for most malaria species (Gendrin and Christophides 2013). Once the mosquito has bitten its mammalian host, the sporozoites infect the liver and form schizonts. When ruptured, these schizonts release merozoites that enter the bloodstream and invade the red blood cells. Some parasites go to the sexual erythrocyte stages and make gametocytes making the vertebrate host infective to a
subsequent mosquito (CDC 2018). Globally, the most threatening species of
*Plasmodium* is *P. falciparum*. This is due to its high prevalence and the severity of
symptoms. This species has a large distribution and is the most common agent of human
malaria (WHO 2016, CDC 2018).

Most of the preventative measures against malaria focus on controlling the adult
mosquito population due to the easy use of insecticides on bed nets and inside residual
spraying. Besides insecticides, more studies are currently focusing on alternative controls
for the mosquito using physiology-based methods, specifically the physiology of the
mosquito midgut. When the *Plasmodium* first reaches the gut, it is at its lowest numbers.
The gut is where the majority of the pathogen development occurs. The first barrier of
infection occurs once the *Plasmodium* is ready to leave the gut and pass through the
epithelium (Ghosh *et al.* 2000). There are many species of Anophelines around the world
with different ecologies and life histories. However, examining the mosquito midgut
provides a common baseline for comparison. The midgut microbiome is one way to
compare.

**Mosquito gut microbiome and bacteria therein**

The gut microbiome has been implicated in affecting a multitude of functions in
humans from diabetes to personality (Gill *et al.* 2006). It has been estimated that humans
have 10 to 100 trillion different microbial cells living in the intestinal tract alone (Gill *et
al.* 2006). Most relevant to my study is that the human gut microbiome has been
associated with immunity (Gill *et al.* 2006). Much like humans, mosquitoes are
considered to have some degree of a mutualistic relationship with their gut microbiota.
While the mosquito microbiome is no doubt much simpler than that of the human microbiome, both start at what is eaten.

Mosquitoes reared in the wild are naturally exposed to a larger diversity of bacteria compared to lab-reared mosquitoes. The microbial community in an adult mosquito’s midgut originates from the food consumed during its larval stages of development (Vantaux et al. 2016). Also known as the gut microbiota, midgut bacteria play an important role in the mosquito’s physiology, including vector competence (Figure 1, Tol and Dimopoulos 2016). Along with vectoral capacity, nutrition the larvae receive during development affects all life history traits such as size, fitness, fecundity, and a strong immune system (Vantaux et al. 2016). It has even been shown that by mitigating the immune response, Anopheles sp. mosquitoes with a lower nutritional uptake have elongated lives from malarial infection (Vantaux et al. 2016). The malarial infection inside the mosquito midgut is considered an “obligate gateway” for infection to be successful (Gendrin and Christophides 2013). This threshold of the midgut epithelial wall is why the bacteria either working with or against the infection are important. With the rise in insecticide resistance, researchers are looking at this interplay between microbiome and vectoral capacity as a possible tool for disease control. In mosquitoes one technique is paratransgenesis, use of a symbiotic organism in the midgut of an organism for the purpose of vector borne disease control.

Seasonality and locality have shown to be important factors in gut microbiome flora of An. gambiae, the main malaria vector in Sub-Saharan Africa (Akorli et al. 2016). Since it has been suggested that seasons and locality have an effect on the microbial composition and the mosquito species type does not have an effect prior to blood feeding,
this information can be applied to other Anopheles spp. around the world (Akorli et al. 2016, Osei-Poku et al. 2012). It is important to notice the differences in bacterial species and quantity for studies that may apply to different regions of the world or in the lab, because although one bacterial species may qualify as a good candidate for mosquito control, in a different geographical area it may not have the desired effect. Understanding the differences between wild and lab microbiomes makes for more cohesive and effective research. If it is found that the wild and lab mosquito microbiome communities are vastly different, techniques could be developed to mitigate the difference. It could change protocols for rearing mosquitoes, such as allowing lab strain mosquitoes to be reared in specific microbial rearing water. Specific rearing environments could be as complicated as the exact same microbes in water for each treatment or using water locally acquired. This cohesive research could translate well in a wild setting and ultimately be more cost effective.

The most common genera of bacteria found in the midgut of Anopheles spp. in order of quantity are Pseudomonas, Aeromonas, Asaia, Comamonas, Elizabethkingia, Enterobacter, Klebsiella, Pantoea, and Serratia (Gendrin and Christophides 2013). However, none of these genera reach one hundred percent prevalence. It is also of note that most of this difference has been shown in studies on lab-reared Anophelines. Comparing the lab protocol of rearing to the natural process of larval development, the available bacteria for consumption differ greatly between the treatments (Boissie et al. 2012, Wang et al. 2011). Due to most of the bacteria being consumed during larval development, adults that have not yet fed on nectar have very high numbers of bacterial operational taxonomic units (OTU) in their guts. The highest diversity is seen during the
larval and pupal stages (Wang et al. 2011). It is thought that prior to blood feeding females must first take a meal from a sugar source such as a flower or fruit to make the flight to their host for blood consumption (Osei-Poku et al. 2012).

The initial loss in diversity through developmental stages is due to melanization during each larval instar. Melanization of the hindgut is induced by prophenoloxidase which is an enzyme that helps fight bacterial infection in the midgut, as represented in the hindgut of *Bombyx mori* (Shao et al. 2012). Compared to other insects, mosquitoes have relatively high OTU counts of bacteria. Often times the total percentage of bacteria is dominated by one genus or even one species (Gendrin and Christophides 2013). An issue that can arise from lab research of the microbiome in mosquitoes is if an abundant bacterium is considered a potential control agent and heavily studied in the lab, it is possible that the abundance of that bacterium would not be the same in the wild. This would then result in the treatment not having the appropriate effect. Although it is difficult to find bacteria for the purposes of paratransgenesis that are effective on all mosquito and malaria species around the world, there is a lot of work being done on a few different species that in combination may be the best answer to the complicated relationship between bacteria and the midgut of the mosquito and the mosquito to the habitat in which it is getting its midgut content.

*Asaia* is an acetic bacterium originally named after Toshinobu Asai, the Japanese researcher who did extensive work on *Acetobacter* (Asai et al. 1964). Acetic acid bacteria can be classified in to four genera: *Acetobacter*, *Gluconobacter*, *Acidomonas* and *Gluconacetobacter* (Yamada et al. 1997). Toshinobu described *Acetobacter* exhibiting peritrichous flagellation, having flagella evenly distributed over the body surface, and the
capability to oxidize acetate (Asai et al. 1964). The family Acetobacteraceae is rod shaped and most strains are motile. *Asaia bogorensis* is the most common Acetobacteraceae that is currently being used for paratransgenesis research in mosquitoes. *Asaia bogorensis* is a gram negative, aerobic, rod shaped, peritrichously flagellated strain of Acetobacteraceae originally isolated from plants, most commonly flowers of the orchid tree, from Bogor, Java, Indonesia (Yamada et al. 2000). Plated *Asaia* colonies are usually pink or yellowish white with a size of about $0.8 \times 10^{-12}$ microns (Yamada et al. 2000). Survival under challenging environments and the ability to oxidize acetate are important factors to consider when understanding the bacteria’s ability to maintain a presence in the midgut of a mosquito throughout its lifetime, including post blood meal when conditions are most acidic.

*Asaia* spp. have been shown as dominant colonizers of *An. stephensi* and, in some cases, appears to be a dependent symbiont (Favia et al. 2007, Chouaia et al. 2012). In the context of this thesis, colonizers are those bacteria that are most commonly found at high concentrations in the midgut. A symbiont is a bacterium that has created a mutualistic relationship with the insect and can be important to the mosquitoes’ physiology. An important factor to consider in any bacteria for paratransgenesis is its ability to exert a direct effect on a pathogen without affecting the fitness as an adult mosquito. Without this, treatment would not be effective in the wild. Under laboratory conditions it has been shown that *Asaia* spp. are localized in the gut, salivary glands, and reproductive organs (Damiani et al. 2010). Favia et al. (2007) showed that in lab-reared *An. stephensi* almost 90% of the entire gut microbiome, as well as other life stages like egg, larvae, and pupae, were dominated by some *Asaia* spp. *Asaia* spp. are also found to be dominant in *An.*
gambiae (Favia et al. 2007). With Asaia spp. dominating the midgut, it is possible that Asaia spp. play a role in insect survival or as a symbiont. It has been shown that when treated with rifampicin, an antibiotic that kills Asaia, the development rate challenged larval survival in An. gambiae (Chouaia et al. 2012). It is possible that certain Asaia spp., especially those that are associated with nectar, would make sense as a symbiont of mosquitoes. Because this bacterium is present in laboratory settings where no wild nectar is available, symbiosis is a possibility.

One of the earliest proof of concept studies of bacterium paratransgenesis as a control for Plasmodium is the use of Pantoea agglomerans. It was shown when effector proteins were unnaturally introduced; parasite development could be blocked in the mosquito gut (Bisi and Lampe 2011). However, researchers have had some breakthroughs in paratransgenesis using an Asaia sp. as a symbiont by introducing a toxin to kill the Plasmodium in the midgut. Bongio and Lampe (2015) showed that two Asaia strains have effectively been modified and used to deliver non-self toxins to inhibit the growth of P. berghei. Those two strains secreted scorpine and antimicrobial peptide anti-Pbs21 scFv-Shival immunotoxin (Bongio and Lampe 2015). Scorpine venom can be attached to Asaia and administered through feeding to adult mosquitoes; the venom would then only be toxic to the Plasmodium. This has worked with An. stephensi with a P. berghei infection under laboratory settings (Bongio and Lampe 2015). With the small evidence of vertical transmission of Asaia, it could be possible to perpetuate these traits throughout several generations.

Enterobacter is another genera of bacteria of interest in mosquitoes. It has been demonstrated that Enterobacter in the midgut can protect Anopheles from Plasmodium
Enterobacter are gram negative, peritrichously flagellated, rod shaped bacteria that can ferment glucose and lactose (Hormaeche and Edwards 1960). A strain of Enterbacter, Esp_Z has been isolated from wild populations and shown to reduce ookinete production by tenfold; the effect is dose dependent (Cirimotich et al. 2011). Esp_Z does this by activation of the insect immune deficiency pathway (IMD pathway), which then increases the transcription of Cecropin1, an antimicrobial peptide, and therefore reduces numbers of Plasmodium (Cirimotich et al. 2011). Operating similarly to Enterobacter, another bacterial genus used for mosquito control is Wolbachia. Effects of Enterobacter on lessening a malaria infection have been based on manipulating the amount of bacteria in a lab. However, these amounts are not at a high enough level in midguts of wild Anopheles sp. (Gendrin and Christophides 2013).

While there has been gut microbiome community analysis work done on the malaria vector, Anopheles, in the lab and in the wild, there is a lack of comparative studies looking at manipulating lab-strain mosquitoes so that their microbiome mimics that of a wild mosquito. If the studies presented above are viable options for use of gut microbial community as malaria preventatives, then understanding the differences in wild microbiome compared to that of a lab rearing protocol environment is key to those efforts being successfully implemented. Wild mosquitoes undergo different types of stresses than lab-reared mosquitoes. One of the stresses is availability of a food source.

Even in a relatively homogenous landscape such as a swamp, there can be microhabitats of bacteria and other food sources not found in other areas. If a bacterium is a successful control agent, it must be plentiful, ubiquitous, and present in both laboratory and wild settings. It is important for the bacterium to provide a more efficient
and reliable control by being present in both the lab and the wild. The trouble with laboratory settings is that they are under “clean” conditions. This allows for possible invasion of a single bacterium species to dominate in the population. From exhaustive literature review, it appears that there has been no comparative analysis of lab strain *Anopheles quadrimaculatus*’ microbiome to its wild type. Likewise, there has been no reported work showing how this possible difference could affect malarial development and transmission. Throughout this thesis it will be discussed how different *An. quadrimaculatus* is in a lab setting versus the wild and discuss how this may affect the current standard for rearing mosquitoes for research and field use.
Figure 1    Interactions between larval habitat and adult life history.

The components shown in boxes represent some of the factors from rearing environment that can contribute to a mosquito’s ability to transmit disease. Ultimately, this complex of factors affect the mosquito’s immune status and overall fitness, two key factors for the vector to transmit disease.
CHAPTER II

INITIAL EXPLORATORY STUDY INTO THE MICROBIOME OF A SMALL SAMPLE SIZE OF MOSQUITOES

Objective I

Through literary research, it was found that there have not been many studies done on the differences in gut microbiome between lab strain and wild mosquitoes. In the same regards there was no attempt to change insectary-rearing protocols to mimic a wild setting to change the microbiome. In order to show proof of concept, the first objective was to determine if there is a difference in midgut microbiome composition between wild rearing and lab-strain reared An. quadrimaculatus. The hypothesis was that since rearing habitats and food availability are different between wild and lab reared mosquitoes, that wild mosquitoes would have a more diverse microbiome. It was also hypothesized that if lab-strain mosquitoes are reared in water from wild habitats that it would result in more consistency between the microbiome of a lab-strain mosquito and a wild mosquito. If the bacteria from lab and wild were not significantly different, this would suggest that lab experiments are reflective of wild rearing.

Materials and methods

For this study wild An. quadrimaculatus adult female mosquitoes were collected in a CDC mini light trap at Sam D. Hamilton Noxubee National Wild Life Refuge. The
trap was left overnight in an area towards the periphery of the swamp prone to flood-like effects. The collection was done on 1 September 2016. From this collection two adult mosquitoes were morphologically and genetically identified and the midguts were dissected. For the lab treatments ORLANDO strain *An. quadrivittatus* were reared under “normal” and “wild” conditions. Normal conditions were the typical lab protocol for rearing this species and wild conditions were this lab strain reared in water collected from the adult trapping site on the same day. Only one mosquito was dissected from the normal conditions and two mosquitoes were dissected from the wild conditions. This gave a total of five mosquitoes analyzed for the 16S analysis. Alpha diversity was measured by using Simpson’s index of diversity and visual distribution of bacteria species.

**General rearing**

For general lab rearing the larvae were hatched together in buckets with deionized water and a small amount of yeast based fish food. The lab strain *An. quadrivittatus* was the ORLANDO strain. Mosquitoes were kept at a humidity around 72% at 27°C and were also on a 12 hour dark/light photoperiod. The finely ground food mixture was ground up shrimp containing no yeast. Once hatched it was assessed if the brood needs to be separated in to another or several other buckets to prevent competition due to overcrowding. Buckets were monitored six days a week and were fed the same food mixture daily. Close monitoring of bacterial growth was done because the yeast base in most of the food product can cause a large outbreak of bacteria that then was harmful to the larval development. In the case of this growth the larvae are removed via pipette or sieve and are placed in a clean bucket with fresh water. This was done at least once
during larval development. The pupae once eclosed, were removed and placed in emergence cages for easy removal of adults at emergence. The rearing of all mosquitoes in the project was done in the same manor except rearing water source. Pupae and emerged adults from each treatment were kept separate. From each trial the larvae were all from the same egg lying period.

**Dissection, identification, and sequencing**

For genetic confirmation of the wild caught mosquitoes Cytochrome Oxidase 1 (CO1) barcoding was used. Two legs from each wild caught mosquito were used for DNA extraction and identification. An Invitrogen PureLink genomic DNA kit for DNA extraction was used. The CO1 primer was used. Together there were three treatments for the PCR: two wild mosquitoes and a negative control. After the DNA was amplified, the product was run on an agarose gel to check for specificity of size. Following that, after cleaning up the product, the samples were sent to Eurofins Operon (Eurofins, USA) for barcoding. Once the results were receive the sequences were run through the BOLD systems database.

Adult mosquitoes from lab strain treatments were dissected 3-5 days post emergence and wild mosquitoes were dissected after collection. Identification for midgut 16S sequencing was done according to Coleman *et al.* 2007 (*Coleman et al.* 2007). For the 16S rDNA microbial community analysis Life Tech PureLink Microbiome DNA purification kit was used for extraction. Illumina-based 16S sequencing was done at fifty thousand reads per sample in the V3-V4 region. For the 16S MiSeq analysis primers 515/806 were used. The forward primer was barcoded. For the PCR, a 28 cycle was used with HotStarTaq Plus Master Mix Kit (Qiagen USA) with the following conditions: 94°C
for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Once the product was amplified, it was checked on agarose gel for intensity of bands. If the PCR product had appropriate intensity and size, then multiple samples were then pooled together and then purified for the preparation of the Illumina DNA library. The sequencing was done at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturers guidelines. Sequences were then joined, barcodes were removed, then sequences <150bp removed, and sequences with ambiguous base calls removed. Sequences were denoised and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a database derived from RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu). All OTU identification was done with the most recent bacterial sequences available at the time of the analysis. Simpson’s index of diversity was performed to analyze the diversity of genera per mosquito.

**Results and Discussion**

Through the CO1 barcoding and Sanger sequencing it is determined that the mosquitoes caught in the wild were *An. quadrimaculatus*. The most common phyla of bacteria found in all mosquito midguts is Proteobacteria followed by Tenericutes and Firmicutes (Figure 2). Lab strain mosquitoes LNOX1 and LNOX2 are dominated by Proteobacteria. Those samples also have more different phyla represented than the Lab strain mosquito reared under normal conditions. The wild mosquitoes, WNOX1 and
WNOX2, have less Proteobacteria and more abundance of other phyla than other samples (Figure 2).

There is an overall number of 130 different genera being represented throughout the samples, however, no samples have all 130 different genera present (Table 1). The top five genera of bacteria represented are *Asaia, Stenoxybacter, Pseudomonas, Candidatus Phytoplasma,* and *Stenotrophomonas* (Figure 3). The lab strain mosquito reared under normal conditions has the least amount of different genera represented, but still maintains a high level of alpha-diversity according to the Simpson’s index of diversity (Table 1). The lab strain mosquitoes reared in wild water have the lowest diversity according to Simpson’s Index of diversity and were heavily dominated by the genus *Asaia* (Figure 3).

Consistent with Gendrin and Christophides (2013), the guts of the mosquitoes appear to be dominated by one bacterial genus. However, in this study, dominance is not represented by the same genera across all samples. It is of note that all lab strain mosquitoes, regardless of rearing habitat, are dominated by *Asaia*. The wild mosquitoes did have Asaia present but in much lower amounts (Table 1). The samples reared in wild water have the lowest Simpson’s index of diversity scores, showing that while the samples have the highest amount of different genera represented, the genera are not of a distribution that is considered diverse or stable.

This exploratory study shows that wild mosquitoes do have a different microbiome than lab-reared mosquitoes. It also shows that it is possible to change the gut microbiome of lab strain mosquitoes by rearing them in wild water. While this change is possible, it is at some cost of allowing specific bacteria to over dominate the midgut. If it
is true like according to Favia et al. (2007) and Chouaia et al. 2012, that \textit{Asaia} is a possible symbiont for some Anopheles species, I propose that it is not \textit{Asaia} being a symbiont, but rather a result of rearing environment that mosquitoes have varying tendencies for \textit{Asaia} growth. \textit{Asaia} is an important bacterial genus to observe and understand how different amounts of it could be changing the physiology of the mosquito. This exploratory study shows that the microbiome from a lab environment is not reflective of the wild type. The difference in rearing environment exhibits the need for further study that is defended in later chapters of this thesis.
Figure 2  Distribution of bacteria according to phylum.

Along the X-axis is the percentage of bacteria represented per mosquito midgut at the phylum level. On the Y-axis are the samples: WNOX2 and WNOX1 are wild collected, LNOX2 and LNOX1 are lab strain mosquitoes reared in wild water, and LAB is a lab strain mosquito reared under normal conditions.
Figure 3  Distribution of bacteria according to genera.

Bacteria here are represented as percent of total bacteria at the genera level. All conditions are the same as Figure 2. The most common bacteria are the darker shades.

Table 1  Table of total OTU, Simpson’s index of diversity, and *Asaia* sp. OTU count.

<table>
<thead>
<tr>
<th>Column1</th>
<th>Lab</th>
<th>LNOX1</th>
<th>LNOX2</th>
<th>WNOX1</th>
<th>WNOX2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total OTUs</td>
<td>69</td>
<td>85</td>
<td>89</td>
<td>83</td>
<td>84</td>
</tr>
<tr>
<td>Simpson's index of diversity</td>
<td>0.839206</td>
<td>0.202158</td>
<td>0.062263</td>
<td>0.819564</td>
<td>0.917029</td>
</tr>
<tr>
<td><em>Asaia</em> count</td>
<td>39864</td>
<td>109931</td>
<td>173341</td>
<td>128</td>
<td>773</td>
</tr>
</tbody>
</table>

This table presents the total operational taxonomic units (OTU) found per sample. It also displays the Simpson’s index of diversity values $D = 1 - (\sum n(n-1))/N(N-1))$ where n is the total amount of individuals of a species and N is the total amount of all individuals. 0 represents no diversity and 1 is perfect diversity. This table also shows the amount of *Asaia* in OTU counts per sample.
CHAPTER III
MICROBIAL COMMUNITIES FROM DIFFERENT REARING ENVIRONMENTS

Objective II

Because of the first objective, a more extensive investigation of how rearing environment effects mosquito gut microbiome was needed. The goal of this objective was to further characterize and define the differences between wild and lab-strain mosquitoes under separate rearing environments. This goal contained two parts. First was an extended study of wild versus lab effects on gut microbiome. And the second was the identification of the Asaia sp. bacteria in the lab strain population.

Methods and materials

Asaia culture/identification

From the investigative study (Goal 1) it showed that Asaia sp. bacteria were more prominent in lab-reared mosquitoes, therefore it was important to identify the possible different in strains found in the laboratory. Identification of Asaia sp. was done by targeting Acetobacteria through selection in broth media according to Yamada et al. 2000 (Yamada et al. 2000). The broth medium contained: 4g D-sorbitol, 0.6g yeast extract, 1g peptone, 0.02g cycloheximide, 200mL DI water, and HCl for getting the pH to 3.5 and finally was autoclaved. Once the broth cooled it was transferred to a sterile plate and 1 mosquito gut, dissected in the same manner as for microbiome DNA extraction from
Mosquitoes were dissected 3-5 days post emergence. After close to a week in the broth, bacteria growth was cultured on media, also according to Yamada et al. 2000, including: 4g glucose, 1.6g yeast extract, 2.4g agar, 1mL ethanol, 1.4g calcium carbonate, and 200mL DI water (Yamada et al. 2000). Plates were incubated at 37°C. Once bacterial growth was noticeable in the majority of plates, colonies were selected based on pink color and then plated again in the same culture media. This process was repeated one more time before final DNA extraction. DNA extraction was carried out using the Invitrogen PureLink Microbiome DNA Purification Kit. After DNA quality and concentration was checked, standard PCR was conducted using the universal 16S bacterial primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1392R (5’-GGTTACCTTGGTACGACTT-3’) following the same conditions for PCR cycle (Srinivasan et al. 2015). PCR product size was checked on agarose gel and after specificity was confirmed, amplicons were sent to Eurofins Operon (Eurofins, USA) for Sanger sequencing. Sequencing results were analyzed using NCBI BLASTn platform against entire nucleotide database. If no identity could be made it was done again against Asaia (taxid: 91914). Identification was determined based on percent identity. The whole process was repeated five times for each mosquito sample. The first three trials for identifying the possible Asaia species in the lab strain mosquitoes were done in August 2017. The second two trials were done in April 2018 after moving to a new insectary.

**Mosquito collection and rearing**

Wild mosquitoes were collected and to reared from three different sites in Mississippi for this study: Noxubee National Wildlife Refuge (NOX), Dorman Lake
(DOR) (Mississippi State University Department of Forestry plot), and a flooded rice field at Mississippi State University Delta Research and Extension Center (DREC) in Stoneville, MS. From these sites three adults were analyzed for their gut microbial content. The collection dates for this experiment occurred in June and July 2017. From these sites semi natural mesocosms (Figure 4) were placed with first instar larvae to develop to maturity. However, natural predators or some other factor killed the mosquito larvae. Because of this water was collected from these sites and brought back to the lab to rear the larvae in. There were a total of 15 different treatments, six of which were water samples, with three mosquitoes each that were used in the microbiome analysis, except LNOX1 where only two mosquitoes were used. Simpson’s index of diversity was used to measure for alpha diversity per mosquito treatment group. ANOVA was performed to confirm if there was any statistical difference between the three mosquitoes in each treatment. A non-metric multidimensional scaling (NMDS) analysis was done to relate the sites and treatments to the bacteria found at the phylum level and then for the top 25 most abundant genera. All mosquitoes reared in the lab were reared in the same manner as the first objective.

A non-metric multidimensional scaling (NMDS) analysis is a non-parametric analysis, which takes non-linear data and reduces it to a few important axis of which then patterns can be interpreted. A NMDS does a rank order correlation, which takes the data and determines the ordination distance compared to the observed dissimilarity. A stress plot is determined to see in the number of axis chosen is appropriate. In order to interpret the data, points that are close together are considered more similar. Similarly if polygons
Results and Discussion

Asaia identification

When initially blasted against the whole nucleotide reserve, the first three trials could not be appropriately identified. This could be due to general inexperience with both bacterial culturing and DNA extraction. Those three trials were then blasted against Asaia (taxid: 91914). The fourth and fifth trials from mosquitoes reared in a new insectary have an identification of Asaia bogorensis when ran again the entire nucleotide reserve (Table 2). The discrepancy in identification could be attributed to improvement in culturing and extraction technique as the study progressed.

Asaia is known as one of the most common bacteria found in the gut of mosquitoes and one of the less abundant bacteria in rearing water (Gendrin and Christophides 2013, Figure 6). More extensive identification criteria needs to be done to confirm the identity of Asaia sp. in the lab. The next step for determining if one of the Asaia sp. would be appropriate for paratransgenesis work would be to see why this specific genus of bacteria propagates well in the midgut.

Microbiome analysis

The ANOVA shows that there is no statistical difference between the three mosquitoes in each treatment. This was true for all treatments (Table 3). Because there is no difference, for the remainder of the analysis for each treatment the average amount of
each bacterium between the three samples is used. There is a total of 27 different phyla identified from the samples. The top five most common phyla are: Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Tenericutes (Figure 5). The stress plot, which represents how many dimensions are appropriate for NMDS analysis at the phylum level, has a score of 0.1033872, indicating that a two-dimensional analysis is appropriate (Figure 6). The final NMDS plot is represented in Figure 7. The NMDS plot shows that lab strain mosquitoes and wild mosquitoes are closely arranged, colored in plum (1-5) and yellow (6-9) respectively, meaning that the two groups share similarities in both species and abundance of the bacteria phyla. Specifically both lab strain and wild mosquitoes share a lot of Proteobacteria and Firmicutes groups. The water samples, in blue (10-15), are not ordinated in space with the mosquitoes meaning that the bacteria types and abundance found in the water is not representative of what will propagate in the midgut of a mosquito (Figure 7, Table 4). Both the LAB (1) sample and LABW (13) samples are ordinated away from the rest of their groups showing that there is a difference in what bacteria is associated with those specific treatments compared to their counterparts (Figure 7).

A total of 823 different bacteria are identified at the genera level for all samples. There is an average of 250 different genera found per treatment (Table 3). The Simpson’s index of diversity indicated that the most diverse treatments are the lab strain mosquitoes under normal rearing conditions and the water samples (Table 3). The lab strain mosquitoes reared in wild water and the wild mosquito treatments all had varying lower index scores. This difference could be due to competition between bacteria in the midgut or the effects of rearing environment. However, since the Simpson’s index of diversity is
based off of both the amount of different species and their abundance in the sample, I do not think that the difference in diversity between the different groups is because of the choice of test.

The findings in the study are constant with Gendrin and Christophides (2013), which is that the most common genera of bacteria found in the mosquito midguts are *Pseudomonas* and *Asaia*. It is represented as OTU counts of each respective genus across all treatments. For both genera of bacteria, neither is comparatively as common in the wild water as they are in the midgut (Figure 8). Interestingly for *Pseudomonas*, with the exception of one treatment (WNOX1), almost all other mosquito treatments have double the amount of *Pseudomonas* than the LAB treatment. The inverse appears to be happening for *Asaia*. While the presence of *Asaia* is much more prevalent in the lab strain mosquitoes reared in wild water and the wild mosquitoes is much larger than in the water samples, the LAB treatment had almost four times as much *Asaia* propagating in the midgut (Figure 8). In these two examples of bacteria, it is showing that the abundance bacteria to propagate in the mosquito midgut are different from what is found in the rearing water. Also that for *Asaia*, rearing habitat can change the amount of *Asaia* that propagates, and that under normal rearing conditions that amount can be quite large.

The NMDS plot at the genera level for the top 25 bacteria found overall is included. The stress plot score for the top 25 bacteria is 0.04908207, indicating that a two-dimensional analysis is appropriate for this data (Figure 9). The top 25 NMDS plot has no overlap, meaning that there is a difference in the bacteria associated with each treatment group (Figure 10). However, all the mosquito treatments, plum and yellow, are more closely related to each other than they are to their rearing water. *Asaia* and
*Pseudomonas* are heavily associated with lab strain mosquitoes. Water from the normal lab conditions (sample number 13) is ordinated far from the other water samples, indicating that rearing water is very different from wild rearing habitats.

Gut microbiome from mosquitoes in this study show that rearing environment can make lab strain mosquitoes more similar to wild mosquitoes but not entirely. The lab strain mosquitoes may have the same number of different bacteria genera, but do not have the same diversity and representation of those bacteria within the gut. Also what it has shown is that despite water from a normal lab rearing environment being vastly different from wild water, lab reared mosquitoes as represented in this NMDS plot are not vastly different from wild mosquitoes. The presence or absence of *Asaia* can affect mosquito fitness (Chouaia *et al.* 2012). Knowing the dynamics of how it so persistent in lab rearing environments could impact its position as a tool for paratransgenesis. In this study’s case, presumably *Asaia bogorensis*, is a good colonizer of the midgut of lab strain mosquitoes reared under normal conditions, but is not overly abundant in wild populations. In order for bacteria to be considered a viable option for paratransgenesis it needs to be both present and dominant in the midgut. If a bacterium is less dominant in a wild mosquito then expected results coming from laboratory research is not realistic.
Figure 4  Semi-natural mesocosms.

Natural mesocosms used to rear wild strain mosquitoes in the wild. Ultimately too many predators could enter so therefore water was collected instead.
Figure 5  Percentage of total bacteria found in the midgut at the phylum level.

Along the X-axis is the percentage of bacteria represented per mosquito midgut at the phylum level. On the Y-axis are the samples: Water samples, wild collected, lab strain mosquitoes reared in wild water, and lab strain mosquito reared under normal conditions. Treatment descriptions in Table 3.

Figure 6  Stress plot for phylum NMDS.

This stress plot represents the observed dissimilarity (X-axis) versus the ordination distance (Y-axis) for the phylum NMDS plot.
Figure 7  NMDS plot for phylum.

Samples 1-5 are lab strain treatments, 6-9 wild mosquitoes, and 10-15 are water samples. A description of samples numbers is in Table 3. In red are each phylum represented in the samples.
Figure 8  Total OTU counts for Pseudomonas and Asaia.

On the top is the abundance of *Pseudomonas* per treatment. On the bottom is the abundance of *Asaia* per treatment. On the X-axis is each treatment and the Y-axis is the OTU count of the respective bacteria.
Figure 9  Stress plot for top 25 genera NMDS.

This stress plot represents the observed dissimilarity (X-axis) versus the ordination distance (Y-axis) for the top 25 genera NMDS plot.
Figure 10  Top 25 genera NMDS plot.

Samples 1-5 are lab strain treatments, 6-9 wild mosquitoes, and 10-15 are water samples. A description of samples numbers is in Table 3. In red are the top 25 represented bacteria in the samples.
Table 2  
*Asaia* identification trials.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Species</th>
<th>Identity</th>
<th>Ran against</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A. sp.</td>
<td>81%</td>
<td><em>Asaia</em> (tax ID: 91914)</td>
</tr>
<tr>
<td>2</td>
<td>A. sp.</td>
<td>82%</td>
<td>Asaia (tax ID: 91914)</td>
</tr>
<tr>
<td>3</td>
<td>A. sp.</td>
<td>82%</td>
<td><em>Asaia</em> (tax ID: 91914)</td>
</tr>
<tr>
<td>4</td>
<td><em>A. bogorensis</em></td>
<td>99%</td>
<td>Whole reserve</td>
</tr>
<tr>
<td>5</td>
<td><em>A. bogorensis</em></td>
<td>99%</td>
<td>Whole reserve</td>
</tr>
</tbody>
</table>

Trials 1-5 of the *Asaia* culture and identification experiment. All identities were done using BLASTn.

Table 3  
Total OTU count, Simpson’s index of diversity scores, and P-values from ANOVA of all treatments.

<table>
<thead>
<tr>
<th>Column1</th>
<th>LAB</th>
<th>LNOX1*</th>
<th>LNOX3</th>
<th>LDOR</th>
<th>LDREC</th>
<th>WNOX1</th>
<th>WNOX2</th>
<th>WNOX3</th>
<th>WDREC</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU count</td>
<td>247</td>
<td>198</td>
<td>268</td>
<td>268</td>
<td>254</td>
<td>248</td>
<td>244</td>
<td>274</td>
<td>254</td>
</tr>
<tr>
<td>Simpson's index of diversity</td>
<td>0.749</td>
<td>0.31143</td>
<td>0.669</td>
<td>0.45</td>
<td>0.5785</td>
<td>0.30404</td>
<td>0.428</td>
<td>0.44379</td>
<td>0.4889</td>
</tr>
<tr>
<td>P-values</td>
<td>0.991</td>
<td>0.75198</td>
<td>0.887</td>
<td>0.847</td>
<td>0.9524</td>
<td>0.87344</td>
<td>0.9807</td>
<td>0.92778</td>
<td>0.9484</td>
</tr>
</tbody>
</table>

| | NOX3W | NOX1W | NOX2W | LABW | DORW | DRECW |
| | OTU Count | 456   | 572   | 458  | 193  | 490   | 406   |
| Simpson's index of diversity | 0.975 | 0.9881 | 0.903 | 0.859 | 0.9365 | 0.88587 |

Represented in this table is the count of different OTUs represented in each treatment. Also represented is the Simpson’s index of diversity $D = 1-(\sum n(n-1))/N(N-1)$ where n is the total amount of individuals of a species and N is the total amount of all individuals. 0 represents no diversity and 1 is perfect diversity. And for the mosquito treatments P-values are shown from the ANOVA to determine if there was a significant difference in microbiome between samples from the same treatment.
Table 4  NMDS plot number assignments for each treatment.

<table>
<thead>
<tr>
<th></th>
<th>LAB</th>
<th>LNOX1*</th>
<th>LNOX3</th>
<th>LDOR</th>
<th>LDREC</th>
<th>WNOX1</th>
<th>WNOX2</th>
<th>WNOX3</th>
<th>WDREC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab strain normal conditions</td>
<td>Lab strain wild Noxubee water</td>
<td>Lab strain wild Noxubee water3</td>
<td>Lab strain wild water Dorman</td>
<td>Lab strain wild water DREC</td>
<td>Wild strain Noxubee</td>
<td>Wild strain Noxubee2</td>
<td>Wild strain Noxubee3</td>
<td>Wild strain DREC</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>NOX3W</th>
<th>NOX1W</th>
<th>NOX2W</th>
<th>LABW</th>
<th>DORW</th>
<th>DRECW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild water Noxubee 3</td>
<td>Wild water Noxubee 1</td>
<td>Wild water Noxubee 2</td>
<td>lab rearing water</td>
<td>Wild water Dorman</td>
<td>Wild water DREC</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

This table shows the number assignments for each treatment in the NMDS plots. This assignment applies to all NMDS plots in the study. This table also shows the treatment descriptions.
CHAPTER IV

IMMUNE GENE RESPONSE IN *AN. QUADRIMACULATUS*

**Objective III**

Another aspect of understanding the microbiome understands how it affects mosquito immunity. For this study, novel immune gene primers were developed for *An. quadrimaculatus*, based on the genes previously shown to respond to bacterial infection in *Anopheles* (Coggins *et al*. 2012).

**Materials and methods**

Mosquito rearing was the same as the previous objectives. Mosquitoes reared in wild water could not be infected with malaria due to small numbers of larvae surviving to adulthood because of predation. The treatments for this experiment were Lab (normal conditions, Lab bloodfed (lab normal conditions after blood feeding), Wild (those mosquitoes caught in the wild), Lab Water (lab strain mosquitoes reared in wild water), and MLab (mosquitoes tested 3-5 days post infection with *P. berghei*).

**Blood feeding and infection**

Blood feeding was done at approximately 5-7 days after emergence. Once the mosquitoes had emerged they were fed a 10% sugar diet and starved for 4-5 hours prior to feeding. For malarial infection a mouse was infected with a strain of *P. berghei* via intraperitoneal injection. Giemsa stain was done to show proof of infection via a tail prick
(Doolan 2012). Confirmation of infection with a blood smear stained with Giemsa was around 3 days post injection. Mice for feeding are anesthetized using a baseline 0.2mL intraperitoneal injection of one part Ketamine 10mg/mL and one part Xylaxine 1mg/mL. At 3-4 after feeding a subset of female mosquitoes were used for RNA extraction.

**Immune gene analysis**

To check for immune status, all mosquitoes included in the study were measured for the relative expression levels of a suite of immune genes. For each treatment there were five biological replicates. Each, quantitative PCR reaction was performed in three technical replicates. If a mosquito was undergoing a physical stress related to malaria or rearing habitat, then at least one of the immune genes should be activated.

Adult mosquitoes from each treatment were collected into TriZol reagent and the RNA extracted, reverse transcribed into cDNA synth with oligo dT. For qRT-PCR two housekeeping genes, RPS7 and RPS17, and representative genes form the major insect immune pathways: Toll, IMD, Prophenoloxidase, Nitric Oxide synthase and include more recently discovered immune pathways involving RNAi, were used to measure immune response. Immune gene selections and primers were based off of Coggins *et al.* (2012) and related to available *An. quadrmaculatus* transcriptome and *Anopheles atroparvus* partial genome sequences via NCBI Primer, BLAST, and VectorBase (Table 5) (Coggins *et al.* 2012). qPCR reactions were accomplished using SyberGreen reagents with an Applied Biosystems StepOnePlus Real-time qPCR system. The holding stage was set at 95°C. The cycling stage was repeated forty times and had the following stages: denaturing 95°C for 15 seconds, annealing 59°C for 30 seconds, and elongation at 72°C for 30 seconds. The melt curve parameters were: 95°C for 15 seconds and 59°C for 1
minute. Standard curves for each primer were created beforehand to evaluate the efficiency of each primer pair using diluted cDNA. To estimate expression levels between mosquitoes under different conditions, ΔΔCt method was calculated following the Applied BioSystem guidelines. An. quadrimaculatus samples reared under standard laboratory conditions were used as a control sample. ANOVA statistics were applied to ΔCt values to compare samples for significant up or down regulation between all immune genes under the different conditions tested here.

Results and Discussion

It was not possible to create a standard curve for the primer Gambicin. That primer in the future will need to be recreated in order to produce more accurate results. Standard curves for all other primers including housekeeping genes are presented in Figure 11. For those genes presented the efficiency (E) is considered appropriate for the study.

For all $2^{\Delta \Delta CT}$ analysis the expression is normalized by the Lab control. This normalizing expression showed defensin has significantly more up regulation in wild caught An. quadrimaculatus compared to the lab (p-value 0.033) and malaria infected (p-value 0.026) mosquitoes according to $2^{\Delta \Delta CT}$ (Figure 12). P-values are based off of ΔCT values that are normalized using the housekeeping genes RPS7 and RPS17. Cecropin and PPO are down regulated in the wild mosquitoes (Figure 12). Defensin, activated mostly in the midgut, elicits a large response in wild mosquitoes (Dimopoulos et al. 1996). This same extreme of reaction is not seen in lab strain mosquitoes reared in wild water. This may point to a more sensitive innate immune system in wild mosquitoes, which may be genetic, and needs to be explored further. Knowing that it is possible to
change the microbiome of lab strain mosquitoes, perfecting the rearing process to control for predation, in order to produce enough mosquitoes for more information to help fill the gap between immune gene expression in wild versus lab strain mosquitoes.
Figure 11  Standard curve for *An. quadrrimaculatus* immune gene primers.

Starting from top left, the immune gene standard curves are as follows: Cecropin (anti-microbial peptide), RPS17 housekeeping gene, Defensin (anti-microbial peptide), RPS7 housekeeping gene, NOS nitric oxide synthase, and PPO (IMD pathway). E is the efficiency value. E is the efficiency value of each primer. Standard curves were constructed using a tenfold cDNA dilution (1,10,100,1000).
Figure 12  Relative expression of *An. quadrimaculatus* immune gene primers.

This graph is based off of the $2^{\Delta\Delta CT}$ which is normalized to the control which are the mosquitoes reared under normal lab conditions. Significance is based off of the ANOVA, which is run using the $\Delta CT$, which is normalized by the housekeeping genes. On the X-axis are four immune genes tested and the Y-axis is relative expression.
Table 5  *An. quadrimaculatus* immune gene primers.

<table>
<thead>
<tr>
<th>Immune Gene</th>
<th>Pathway</th>
<th>Immune Gene Abbreviation</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prophenoloxidase forward</td>
<td>Immune deficiency</td>
<td>PPO</td>
<td>AGCTGTCTACTACATGCAC</td>
</tr>
<tr>
<td>Prophenoloxidase reverse</td>
<td>Immune deficiency</td>
<td>PPO</td>
<td>TATCCCTCAACCCATATTCA</td>
</tr>
<tr>
<td>Ribosomal protein 7 forward</td>
<td>Housekeeping</td>
<td>RPS7</td>
<td>GTGCTGGAAGATTTGGTCTTC</td>
</tr>
<tr>
<td>Ribosomal protein 7 reverse</td>
<td>Housekeeping</td>
<td>RPS7</td>
<td>TCGATGTTGCTGCTGTTCT</td>
</tr>
<tr>
<td>Cecropin forward</td>
<td>Anti-microbial peptides</td>
<td>Cecr</td>
<td>CTCTGCTTTTTGCTTTGC</td>
</tr>
<tr>
<td>Cecropin reverse</td>
<td>Anti-microbial peptides</td>
<td>Cecr</td>
<td>TTCTCTGCGGCGCTTTAAAC</td>
</tr>
<tr>
<td>Ribosomal protein 17 forward</td>
<td>Housekeeping</td>
<td>RPS17</td>
<td>AGGCTATCTGGAGCTGGAG</td>
</tr>
<tr>
<td>Ribosomal protein 17 reverse</td>
<td>Housekeeping</td>
<td>RPS17</td>
<td>CGCAATGAAACTACGCTCTAC</td>
</tr>
<tr>
<td>Nitric oxide synthase forward</td>
<td>RNAi</td>
<td>NOS</td>
<td>GAAAGGATCGATTATAAGACCCG</td>
</tr>
<tr>
<td>Nitric oxide synthase reverse</td>
<td>RNAi</td>
<td>NOS</td>
<td>TCCAGAAACGGGTCAACAG</td>
</tr>
<tr>
<td>Defensin forward</td>
<td>Anti-microbial peptides</td>
<td>Def</td>
<td>AGGAAGCTGACGCTCCATGCTC</td>
</tr>
<tr>
<td>Defensin reverse</td>
<td>Anti-microbial peptides</td>
<td>Def</td>
<td>CAGAGCGGACACCGACCTATC</td>
</tr>
<tr>
<td>Gambicin forward</td>
<td>Anti-microbial peptides</td>
<td>Gamb</td>
<td>CGAGCGCGGATGCAATTTGAAATG</td>
</tr>
<tr>
<td>Gambicin reverse</td>
<td>Anti-microbial peptides</td>
<td>Gamb</td>
<td>TATCTCCTAACTGCAACCGCACTGC</td>
</tr>
</tbody>
</table>

This table contains the primer strands used for each immune gene as well as the pathway it utilizes. Also included is the abbreviation that is used for each immune gene for this experiment.
REFERENCES


