COLD TOLERANCE OF NATIVE *WOLBACHIA* ENDOSYMBIOTES IN AEDES ALBOPICTUS LARVAE

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Submitted to the

Faculty of the College of Arts and Sciences

of American University

in Partial Fulfillment of

the Requirements for the Degree of

Master of Science

In

Biology

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April 30, 2019

Date

2019

American University

Washington, D.C. 20016

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ABSTRACT

This series of experiments investigated the cold tolerance of native *Wolbachia* endosymbiotes in *Aedes albopictus* larvae. *Ae. albopictus* was exposed to a low temperature treatment at various larval instars to determine if low temperature affects the density of the native *Wolbachia* strains. Quantitative PCR was used to generate density estimates of the two strains of *Wolbachia* in larval mosquito samples from three populations collected in the United States. These density estimates were used to determine if there is a significant difference in *Wolbachia* density due to cold treatment. The results showed no difference in density between larvae raised at 20℃ or 10℃ for seven days. This was true for each population tested. This data is useful in the context of *Wolbachia*-based mosquito control methods, which are currently being developed for use against *Ae. albopictus* because it is a global disease vector.

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CHAPTER 1

INTRODUCTION

Aedes albopictus

The global spread of the mosquito *Aedes albopictus* is a significant public health issue (Armstrong, et al, 2017). This species is a vector for a wide variety of Arboviruses; including Yellow Fever Virus, Dengue Fever Virus, Chikungunya Virus, and Zika Virus (Ruiling, et al, 2018).Ae albopictus has many traits that increase its effectiveness as a vector. It is a container breeder that thrives in human altered habitat, feeds during the day, and most importantly takes multiple blood meals per oviposition cycle (Kraemer, et al, 2015). Its natural range in Asia includes tropical and subtropical regions, allowing this species to survive in a wide variety of climates (Fredricks, et al, 2015). This, combined with the global tire trade, has allowed this species to spread across the world in the last 30 years. Its range now includes significant portions of North America and Europe. In these regions, *Ae. albopictus* poses a serious public health threat because of its ability to spread disease. In Europe, there is a high possibility of future Dengue outbreaks, and *Ae. albopictus* will likely play a role in them (Schaffner, et al, 2014).

Wolbachia

In order to counter these invasions, new mosquito control techniques are being developed. One of the most promising strategies involves using bacteria from the genus *Wolbachia* (Flores, et al, 2018). These bacteria are well known for their ability to manipulate host reproduction (Jiggins, 2017), including an effect known as cytoplasmic incompatibility. Cytoplasmic incompatibility (CI) is caused by an infection of the hosts' reproductive tissues by

Wolbachia (King, et al, 2018). In females, *Wolbachia* localize in germ line tissue during development and thus are inside of egg cells as they develop (Serbus, et al, 2008). In males, *Wolbachia* bacteria infect sperm generating tissues, causing changes in sperm cells that prevent them from producing viable embryos (Ruang-Areerate, 2004). The sperm are modified so that the paternal chromosomes will fail to segregate properly during meiotic anaphase post fertilization (Dobson, et al, 2001). This change can be reversed, or "rescued," inside of an egg cell during fertilization, if that cell has the same strains of *Wolbachia* that were present in the male. Therefore, rescue is strain dependent. In nature, most *Ae. albopictus* are infected with *Wolbachia*, with typically over 99% of field collected *Ae. albopictus* testing positive for its presence (Noor Afiza, et al 2015; Ruang-Areerate et al, 2008; Tortosa, et al, 2010; Tsai, et al, 2017). *Ae. albopictus* is naturally infected with two strains of *Wolbachia*: wAlbA and wAlbB. *Wolbachia* makes up a large percentage of mosquito bacterial microbiome (Hegde, et al, 2018). wAlbA typically exists at a much lower density than wAlbB, but neither seems to impact the density of the other (Mounton, et al, 2003). Both strains can produce CI independently, but together they produce a bidirectional CI.

Research into *Wolbachia* is critical to improve its effectiveness as a mosquito control technique. There are many differences in the characteristics of the various Wolbachia strains. Strains display a wide range of reproductive phenotypes and also have been shown to possess different viral blocking capabilities (Werren, 1997; Lu, et al, 2012). Different strains also have significantly different thermal preferences (Ross, et al, 2017). The genetic and molecular differences responsible for these different traits are not well understood (Osbourne, et al, 2012). Thus research into the traits that specific strains possess is necessary to gain better understanding of *Wolbachia* mechanisms and their effects.

Another important strain-related trait is bacterial load or density. *Wolbachia* density is important for two reasons: (1) certain density levels are required for successful maternal transmission of *Wolbachia*, (2) sufficient density is required in order to see viral blocking effects (King, et al, 2018). This makes studying bacterial density important to determining the effectiveness of *Wolbachia* control strategies. Temperature, diet, and age have all been shown to play important roles in *Wolbachia* density (Mouton, et al, 2007). Different populations of mosquitoes may significantly different average *Wolbachia* loads (Tortosa, et al, 2010). Analyzing the determinants of *Wolbachia* density is crucial to using the bacteria in mosquito control, since the technique relies on miantainining stable densities in the wild (Flores, et al, 2018).

Factors affecting *Wolbachia* Density

Diet can have particularly significant impacts on *Wolbachia* density in mosquitoes. At the larval stage, mosquito growth is highly dependent on food availability (Dutton, et al, 2004) and *Wolbachia* appears to use host cell division to replicate itself (Pietro, et al, 2016). *Wolbachia* has a reduced genome and competes with its host for amino acids (Caragata, et al, 2014). *Wolbachia* also relies on its host lipid metabolism (Molloy, et al, 2016). Therefore, feeding at the larval stage likely has a significant impact on *Wolbachia* density, because this is a period of rapid growth and cell division for the mosquito. Higher larval rearing densities have been linked to lower *Wolbachia* loads, which also suggests *Wolbachia* is negatively affected by limited nutrition (Wiwatanaratanabutr and Kittayapong, 2009). Blood feeding adult mosquitoes may also cause significant increases in *Wolbachia* density. In Ae aegypti, a single blood meal has been shown to increase *Wolbachia* density (Amuzu, et al, 2016). High-protein and high-sugar diets

have been shown to have significant impacts on *Wolbachia* density in Drosophila melanogaster (Ponton, et al, 2014). *Wolbachia* density also seems to vary by age. In *Ae. albopictus*, both strains of *Wolbachia* show sex and age specific trends (Tortosa, et al, 2010). In males, wAlbA density is negatively correlated with age and seems highest at emergence (Calvetti, et al, 2016). Females show a positive correlation between wAlbA density and age (Calvetti, et al, 2016). In males, mean wAlbB titer was shown to increase with age (Calvetti, et al, 2016). Females showed a more complicated trend, with mean wAlbB titer increasing in females during days 10-15 and decreasing in females at 19-21 days (Calvetti, et al, 2016).

Temperature Impacts On *Wolbachia*

Temperature has been repeatedly shown to play a role in *Wolbachia*-host relationships (Thomas, et al, 2003). There is evidence that climate and season conditions can impact *Wolbachia* density, and there is evidence that exposure to certain temperatures can impact it as well. The impact of temperature on *Wolbachia* has been studied in several strains, including those found in *Ae. albopictus*. Past studies have typically focused on the impact of high temperature on *Wolbachia* load because this bacteria used in mosquito control programs in the tropics. There are a few studies which do examine the impact of low temperature on this genus of bacteria.

High temperature has been shown to reduce *Wolbachia* density in many species. In Ae. aegypti, exposure to high temperatures during adulthood can significantly reduce *Wolbachia* density (Ulrich, et al, 2016; Ross, et al, 2017). This was particularly true when females were raised in these conditions from their third or fourth instar. In species of *Trichogamma* wasps, exposure to temperature of 31°C can completely eliminate *Wolbachia* infection (Pintureau and

Bolland, 2001). Attempts to increase the thermal tolerance of *Wolbachia* strains in Ae. aegypti through selection have proven unsuccessful (Roos and Hoffman, 2018).

The possible effect of low temperature on *Wolbachia* density can be seen in regional comparisons. Studies on D. melanogaster in Australia suggest a relationship between *Wolbachia* infection density and cline (Kriesner, et al, 2017). Tropical regions averaged higher infection frequencies than temperate ones. In the butterfly *Zizeeria maha*, *Wolbachia* density was shown to vary seasonally, with high densities in the spring and low densities in the fall (Sumi, et al, 2016). This has been suggested as evidence of a temperature impact on *Wolbachia* density. In *Drosophila innubila*, *Wolbachia* densities were on average 3-5 times greater in flies caught in the spring than in the fall (Unckless, et al, 2009). There is also evidence that *Wolbachia* infection changed thermal preferences in *Drosophila melanogaster* (Truit, et al, 2017). Thus there is evidence that natural *Wolbachia* density may be affected by low temperature.

Acute exposure to low temperatures can also impact *Wolbachia*. Exposure to low temperature has been shown to lower density in some species and increase it in others. In *Drosophila melanogaster* exposure to low temperature was correlated with increased *Wolbachia* densities (Moghadam, et al, 2018). In the wasp *Nasonia vitripennis*, *Wolbachia* density was significantly lowered by a cold treatment of 18℃, particularly when the exposure began in the larval and pupal stages (Bordenstein and Bordenstein, 2011). In *Ae. albopictus*, a temperature treatment of 10℃ on adults has been shown to reduce *Wolbachia* density of both strains (Tsai, et al, 2017). This experiment reported that incubating *Ae. albopictus* adults at 10℃ had little impact on *Wolbachia* infection rate after 10 days, but by day 15 a significant decline in infection rate was observed. Another study found that raising *Ae albopictus* at 18℃ at several different life stages can lead to a slight increase in *Wolbachia* load, possibly caused by a population decline in

other bacterial species (Guégan, et al, 2016). In its global distribution *Ae. albopictus* survives in a wide range of temperature conditions, and *Wolbachia* density may vary in a temperature dependent manner across that range. In addition, different *Wolbachia* strains may have different cold-tolerances.

Experimental Design

The goal of this experiment is to determine the relative cold tolerance of the two *Wolbachia* strains commonly found in Ae albopictus. In order to test this larvae from three populations of *Ae. albopictus* will be grown under two temperature regimes. These samples will be used to determine if the cold treatment has a significant effect on *Wolbachia* load. The temperature chosen for the cold treatment, 10℃, was selected as it is at the low temperature survival threshold of *Ae. albopictus*. The comparison temperature, 25℃, represents a typical *Ae. albopictus* rearing temperature. The cold tolerance of both strains will be analyzed, as each may have its own thermal preferences. The populations will also be analyzed to look for significant differences in cold tolerance between them. The populations come from three geographic locations with distinct climatic factors, Ohio, South Carolina, and Mississippi. While all populations may be occasionally exposed to temperatures of 10℃, there may be differences in how well *Wolbachia* in each of these populations handles the cold treatment, since they experience different climates in nature. This project will also test some samples from different larval instars, and will test the effect of increasing the duration of the cold treatment. Testing of different instar stages might show some significant differences in either average *Wolbachia* load or the cold tolerance of *Wolbachia* in different developmental stages. The results of these tests

may provide some evidence for the impact of development on *Wolbachia* replication. Since the *Wolbachia* are likely replicating in coordination with host cell division, it is possible that as the mosquito larva grow and develop this is causing their *Wolbachia* load to increase. The duration test will provide some evidence about whether the effect seen after 7 days remains stable at 14 days.

CHAPTER 2

METHODS

Mosquito Samples:

Three populations of Aedes albopictus were used in this study. Samples were collected in August of 2017 from Colombia, South Carolina, Columbus, Ohio, and Pontotoc, Mississippi. F4 eggs from each of these populations,were obtained from Dr. Peter Armbruster (Georgetown University). The eggs were reared and used for cold tolerance experiments. In the first experiment, all populations were used to generate data. For the duration and instar experiments, only eggs collected from Columbus and Columbia were used.

Mosquito Rearing

Mosquito eggs were hatched in 100 ml petri dishes containing 60 ml of deionized water. Sections of egg laying paper were cut off and submerged in the water for hatching. Two dishes per population were established. The dishes were kept in a long day cycle of 16 hours of light and 8 hours of darkness. The air temperature was a relatively constant 20°C. The ambient humidity was raised to approximately 60%. Mosquito dishes were fed 50 ml of 1g/50ml tetra fish flake solution once per week, delivered by pipette. Once in the plate well, mosquito larvae were given 5ml of the same solution for food once at the start of the trial. Samples studied for two weeks received an additional feeding of 5ml at the beginning of the second week of treatment.

Experimental Plates

During each experiment mosquito larvae were placed in 24 well plates. One larvae was placed in each well. In order to stock the plates, first a large group of suitable instar larvae were collected from each colony dish. From these, larvae were randomly selected for placement into

wells. In the first experiment fourth instar larvae were used. Four plates were made per colony source. Each set of four plates contained samples from two colony dishes. Two plates per colony were placed in the fridge, while the other two were kept under normal conditions. In the second experiment, two plates were created per population. Both came from the same colony dish.

Mosquito Treatments

The cold treatment mosquitoes were placed in a fridge at a constant temperature of 10℃. The control conditions consisted of development in an ambient environment at 25℃. Both treatments were carried out under 24-hour day cycles in order to minimize temperature fluctuations. Ine the first experiment, all samples were treated for one week. In the second experiment some samples were kept in treatment for two weeks.

Sample Sacrifice:

In the first experiment, half of all wells were randomly selected for sacrifice after one week. In the second experiment, during the first week, 24 wells were randomly chosen for sacrifice. Six samples were collected from each plate. After two weeks, four more samples were randomly selected to be sacrificed per plate.

***Wolbachia* Density Quantification**

Wolbachia density in Ae albopictus larvae and adults grown under different temperature conditions was quantified using Quantitative PCR. The wAlbA strain was quantified using the primer set 328F(CCAGCAGATACTATTGCG) with 691R(AAAAATTAAACGCTACTCCA).

Primer set 183F(AAGGAACCGAAGTTCATG) with 691R(AAAAATTAA ACGCTACTCCA) was used to amplify wAlbB 44 . The CT numbers for these primers were normalized using the CT value for the Homothorax gene, using a primer set of F(TGGTCCTATATTGGCGAGCTA) and R(TCGTTTTTGCAAGAAGGTCA). qPCR reactions were carried out with a volume of 10ul, each using 3µl of 10ng/µl gDNA from each sample along with 0.5µl forward and 0.5µl reverse primers and 0.15ul tracking dye. The qPCR machine used a thermal program of 95°C for 5 mins, 40x cycles at 95°C for 15 sec and 60°C for 30 sec, followed by a melt-curve analysis.

Density Data Analysis:

The *Wolbachia* load of each strain in each sample was quantified using the threshold cycle (Ct) values obtained from the qPCR results. Each sample had three technical replicates per gene. The median of these Ct values was used as the estimate for the true Ct of that sample. Then the relative loads were quantified for each strain by subtracting the Ct value for the control gene HTH from the Ct value for each strain specific primer. This variable, which represents how many more cycles numbers it took for the strain specific genes to amplify, was termed ΔCt. The higher the ΔCt, the less bacterial cells present in the sample. Data was uploaded into R studio using a custom data entry script. Data analysis was also done with a custom script in R. Both are included in the supplemental information for this paper. This script was used to do statistical analysis on the data to determine the significance of different variables.

CHAPTER 3

RESULTS

The results from the first experiment did not show a significant differences in mean ΔCt for either strain of *Wolbachia* in *Ae albopictus*. The second experiment provided data which indicates potential significant differences based on treatment duration, and larval instar. Table 1 summarizes the data regarding the number of samples tested divided by instar, treatment, and population. This experiment tested 65 samples. 30 samples were exposed to the cold treatment, while 35 were grown in normal conditions. These samples consisted of 45 fourth instar, 15 third instar, and 5 second instar larvae. 20 of the samples tested were from Columbus, 30 samples were from Colombia and 15 samples were from Pontotoc. The average ΔCt for strain B was 2.59, compared to an average ΔCt of 3.42 for the A strain. This indicated on average more wAlbB cells present in the samples than wAlbA. A relatively higher ΔCt is an indication of a lower *Wolbachia* load.

Table 1: Samples Tested

Population	4th Instar 10°C	4th Instar 20°C	3rd Instar 10°C	3rd Instar 20°C	2nd Instar 10°C	2nd Instar 20°C	Totals
Columbia, SC	7	9	8	7	0	0	31
Columbus, OH	6	7	0	0	2	3	18
Pontotoc, MS	7	9	0	0	0	0	16
Totals	20	25	8	7	2	3	65

Table 1 contains details about the number of samples tested in each treatment, population, and instar

Figure 1: Effect 10°C Treatment ΔCt. Results are organized by strain.

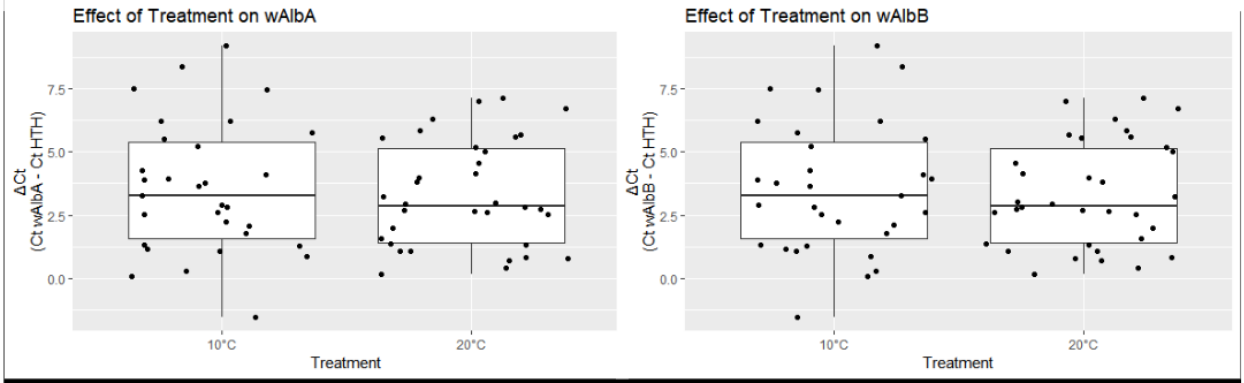

Figure 1 displays the effect 10°C treatment ΔCt. Results are organized by strain. N=65. For wAlbA, mean ΔCt in 10°C treatment was 3.542, and in the 20°C treatment mean was 3.314, yielding a p-value of 0.699. For the wAlbB, mean ΔCt in the 10°C treatment was 2.620, and in the 20°C treatment mean was 2.571, yielding a p-value of 0.856.

There was no evidence of a difference in ΔCt between 10°C and 25°C grown larvae. Figure 1 is a box and whisker plot showing the ΔCt values for both of the strains of *Wolbachia* in both growing conditions. Figure 2 further breaks this data down by population. Welch's two sample t-tests found no evidence of a significant difference in means between treatments in any populations. Table 2.1 contains the means and p-values associated with these tests. The treatment effects were also measured for each larval instar. No instar showed a significant difference in mean between treatments for either strain. The results of these tests are displayed in Table 2.2.

Table 2.1: Mean ΔCt by Population						
Population	wAlbA			wAlbB		
	Mean 10°C	Mean 25°C	P-value	Mean 10°C	Mean 25°C	P-value
Pontotoc	4.877	4.447	0.692	3.264	3.040	0.576
Columbia	3.196	2.857	0.697	2.741	2.625	0.765
Columbus	3.08	2.954	0.951	1.918	2.069	0.770

Table 2.1 displays the mean ΔCt by population, divided by treatment and strain. Welch's two sample t-tests were done for each population to test for significant differences caused by treatment. The associated p-values for each comparison are also given

Figure 2: Effect of 10°C Treatment on ΔCt, by Population

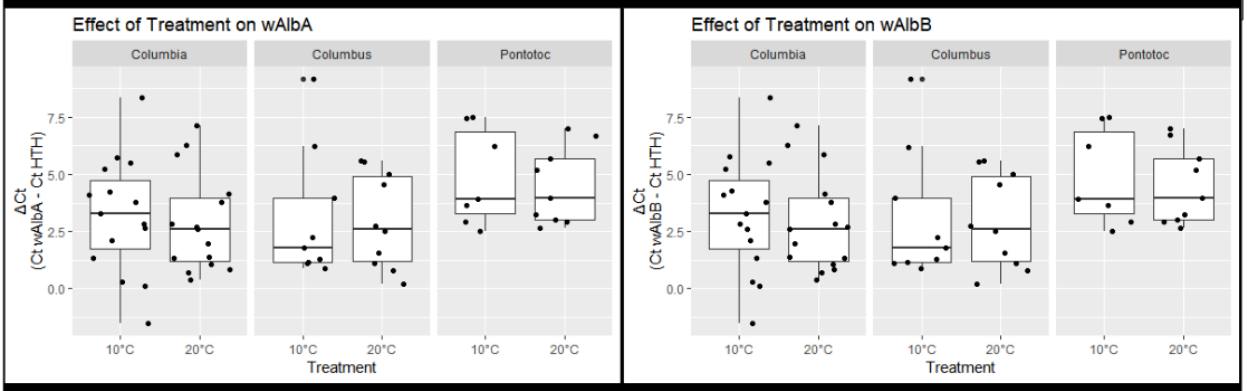

Figure 2 displays the effect of the 10°C treatment on ΔCt, divided by population and strain. None of the populations showed a statistically significant difference in mean ΔCt between treatments. P-values for these tests ranged between 0.576 and 0.951. N=31 in Columbia, N=18 in Columbus, and N=16 in Pontotoc.

Table 2.2: Mean ΔCt by Instar						
Instar	wAlbA			wAlbB		
	Mean 10°C	Mean 25°C	P-value	Mean 10°C	Mean 25°C	P-value
4th	4.472	3.585	0.188	2.744	2.741	0.991
3rd	1.707	1.967	0.814	2.395	2.405	0.986
2nd	1.115	4.209	0.063	2.215	1.600	0.318

Table 2.2 displays the mean ΔCt by instar, divided by treatment and strain. Welch's two sample t-tests were done for each population to test for significant differences caused by treatment. The associated p-values for each comparison are also given.

Population Results

Anova modeling was done to test for a relationship between population and ΔCt for either strain. Initially population was highly significant (alpha = 0.5) in relationship to wAlbA and wAlbB density, but Tukey adjustments revealed only three possibly significant values. In the A strain, only the difference between Pontotoc and Columbia was marginally significant (p-value = 0.057). B strain showed significant differences in Pontotoc-Columbus (p-value = 0.0030 and Columbus-Columbia (p-value = 0.057). The data passed homogeneity of variance test assumptions, however, normality of distribution was violated in the wAlbA. In order to account for this a Kruskal-Wallis rank sum test was performed. In the case of both strains a significant

result was not found (p-value = 0.476).

Figure 3: Differences in wAlbB ΔCt by Population

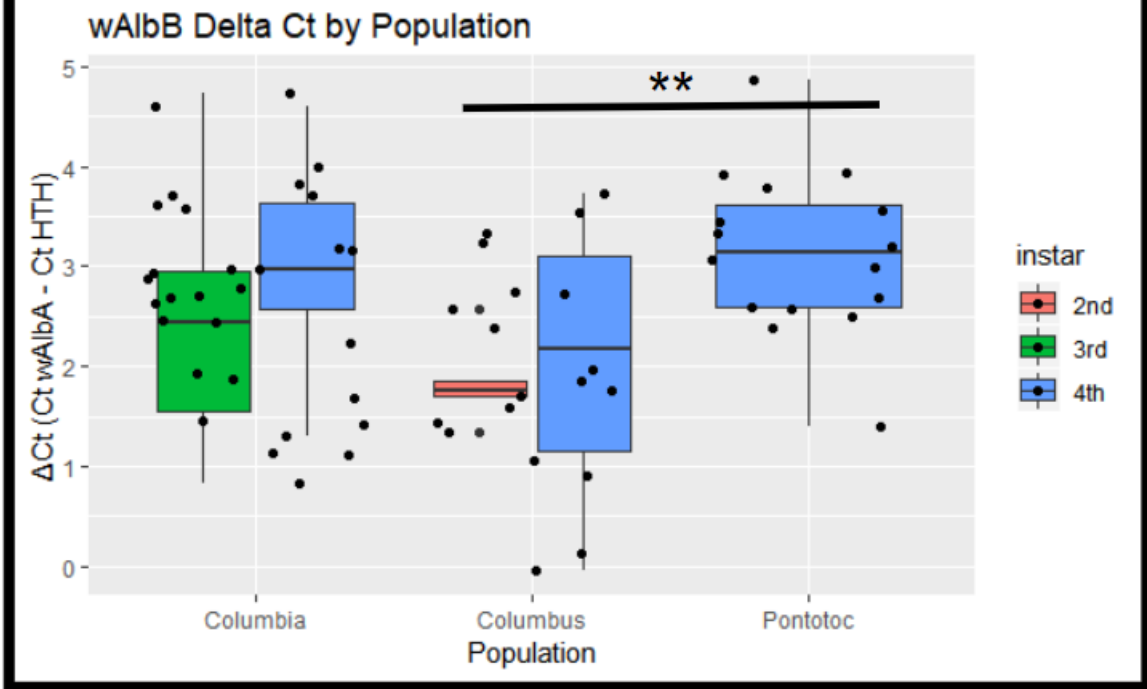

Figure 3 shows differences in ΔCt by population. B strain showed significant differences in Pontotoc-Columbus (p-value = 0.0030) and a slight difference Columbus-Columbia (p-value = 0.057)

Instar Results

The second part of this experiment generated data about *Wolbachia* load in different larval instars. Figure 3 displays the results of the experiment based on instar in each strain. Anova modeling was done on this data to test for significant differences in the mean ΔCt of either *Wolbachia* strains in the instars. Initial tests showed a significant relationship between A strain and instar (p-value = 0.005, which was confirmed to be significant post Tukey adjustment

(4th-3rd instar, p-value = 0.003). The data passed formal analysis for homogeneity of variation and normality. Figure 3 presents the results of this test.

Figure 4: Differences in wAlbA ΔCt by Instar

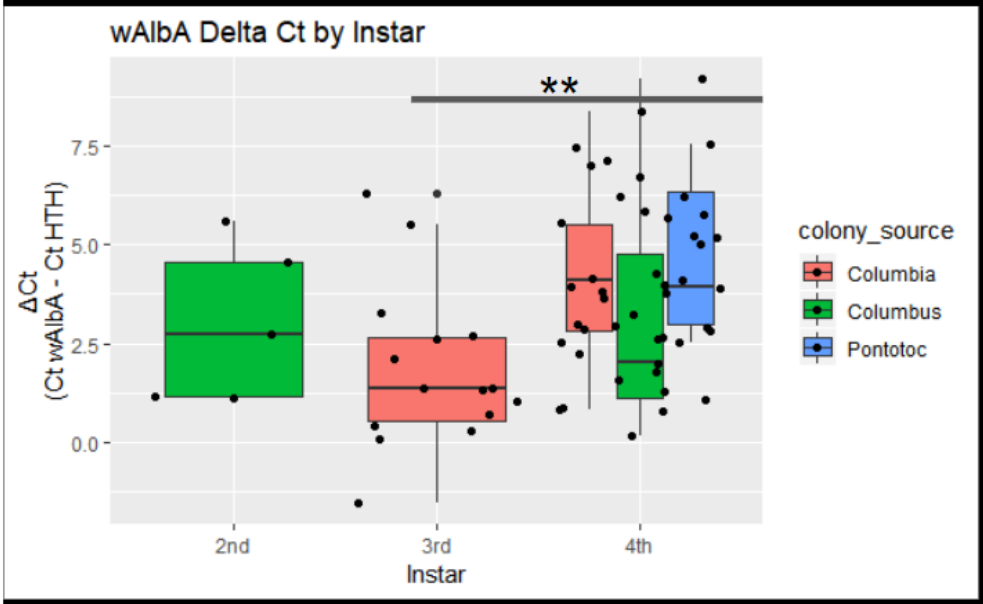

Figure 4 shows differences in ΔCt by instar. The difference between the mean ΔCt for fourth and third instars was found to be statistically significant, yielding a p-value of 0.003. The difference in ΔCt was estimated to be 2.170. The samples sizes were 45 fourth instar, 15 third instar, and 5 second instar.

Instar-Population Interaction

In order to test the potential interaction between instar and population, two-way ANOVA testing was conducted. An additive model was chosen since there was no interaction to directly test. Testing done on wAlbA showed a significant effect of instar on the model (p-value = 0.004). wAlbB saw a significant impact from population (p-value = 0.009). Tukey adjustments of this data revealed only a difference between fourth and third instars in wAlbA (p-value = 0.003). None of the adjusted population effects were significant. A type 3 Anova sum of squares

test was then implemented. It returned a significant difference between instars in wAlbA (p-value = 0.001), and between populations in wAlbB (p-value = 0.009). Interpretation of this data is complicated because wAlbA was not normally distributed, and wAlbB is close to violating the homogeneity of variance rule (p-value = 0.056).

Treatment Duration Results

Data from the second experiment showed some significant effects from extending the cold treatment. Figure 5 depicts group means of the ΔCt between 7 and 14 day treated samples broken down by instar. Within the Colombia samples, all of which were 3rd instar, a significant difference was detected in wAlbA between the the 7 and 14 day samples (p-value = 0.014). No other samples showed a significant impact from treatment duration.

Figure 5: Effect of Duration on ΔCt by Instar

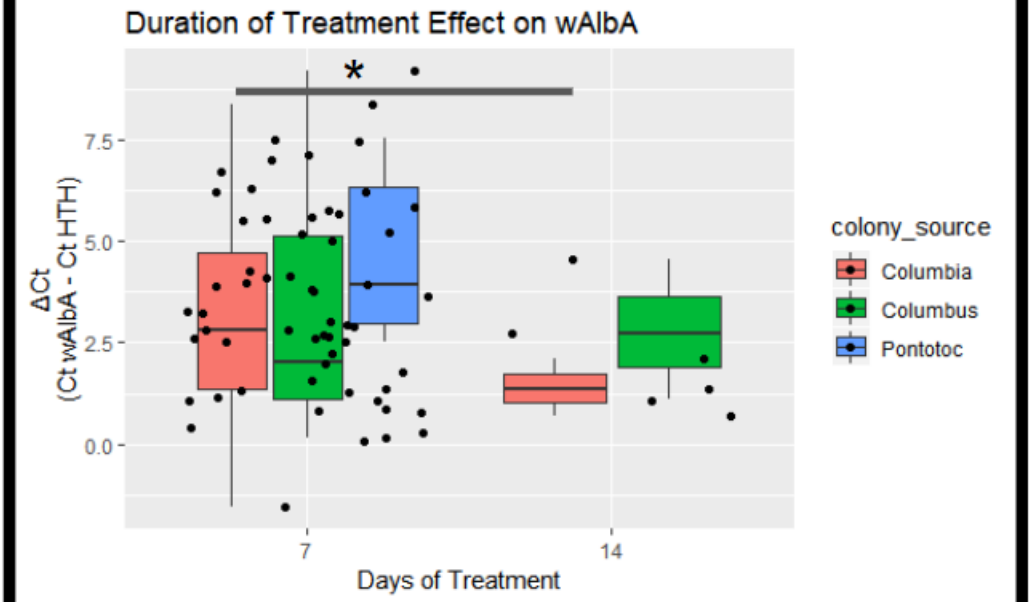

Figure 5 shows the results of the duration tests. Results for wAlbA in Colombia samples had an estimated for mean ΔCt at 7 days was 3.210, while at fourteen days mean ΔCt was 1.370. This yielded a p-value of 0.0146. Sample size was 27 for seven days and 3 for 14 days.

Statistical Checks

Statistical analysis was used to look for biases (Supplemental figures). The effect of date sacrificed, plate, and well were modeled using anova and none had a significant p-value. A figure was constructed comparing the median and mean values for ΔCt to support using the medians as a proxy for the true Ct. Linear regression was also performed to show low correlation between concentration and either sample number or ΔCt.

CHAPTER 4

DISCUSSION

Cold Treatment

The main purpose of this experiment was to investigate the effects of a 10℃ cold treatment on *Wolbachia* load in *Aedes albopictus* larvae. The results indicate that this treatment did not effect *Wolbachia* load. This was true for both strains wAlbA and wAlbB, indicating that both strains have similar cold tolerances. 10℃ was chosen for the cold treatment because it is at the lower bound of temperatures where *Ae. albopictus* larval development can occur (Chang, et al, 2007). Development was noticeably slower in the cold treated samples. The fourth instar larvae selected for the first round of experiments continued to develop normally in the control conditions. In the group exposed to the normal conditions, four adults emerged and fourteen pupae formed during the seven day treatment. In contrast, 14 larva developing at 10℃ had pupated by the end of the trial, but no adults had emerged. Additionally, the refrigerated samples suffered higher mortality, with 22 dying during the trial compared to one in the normal condition plates. This indicated that growth and development may have slowed in these larvae, and perhaps the *Wolbachia* inside them were experiencing the same effect. However, the testing showed no significant difference between *Wolbachia* load caused by larvae developing for 7 days at 10℃.

The results fit in well with *Ae. albopictus* biology. *Aedes albopictus'* natural range includes areas which experience similar cold temperatures (Armstrong, et al, 2017). If the native *Wolbachia* endosymbionts carried by *Ae. albopictus* could not withstand these temperatures, then northern populations would produce fewer viable offspring due to cytoplasmic incompatibility between individuals in them who may or may not have viable *Wolbachia* infections. This cold

tolerance allows for *Wolbachia* to spread with its host far up into its northern range. If all populations harbor bacteria with similar cold resistance, then they all have the potential to spread into cold regions with their hosts.

Comparative genomic studies may yield some insight into the differential cold tolerances of different strains of *Wolbachia*. While the two strains carried by *Ae. albopictus* appear to be cold tolerant, other strains have been shown to decrease in density when exposed to low temperatures (Bordenstein and Bordenstein, 2011). Recent attempts to selected for heat tolerance among mosquitoes have proved unsuccessful (Roos and Hoffman, 2018). Therefore, finding strains and the genes they carry which grant them cold or thermal tolerance may be essential to designing strains for *Wolbachia* control programs.

Population

The experiment also examined colony source in order to determine if any of the populations used had significantly different ΔCt values for either strain. The results are complicated because not every population had multiple instar stages tested. However, the significance value of the result (p-value 0.003) is evidence that the difference is real. This evidence of difference in *Wolbachia* load between populations came from analyzing the A strain mean ΔCt between Pontotoc and Columbus. This is interesting because these two populations experience very different climatic conditions in nature, which may have affected their *Wolbachia* loads. Columbus, Ohio experiences more low temperature days than Pontotoc, Mississippi. Previous research surveying different populations of *Ae. albopictus* for the density of wAlbA and wAlbB have found significant differences between average Wolbachia load between populations (Tortosa, et al, 2010). Other organisms have seen differences in *Wolbachia* load based on temperate and tropical climatic conditions (Kriesner, et al, 2017). Further testing should be done,

including examinations of more populations from a wider range of climates should be examined in order to determine if this is a real effect.

Instar and Duration

This project was also designed to obtain data on differences in *Wolbachia* load that may exist between different larval stages. The evidence is very limited by sample size, with only 15 third and 5 second instar larvae tested. The results did indicate, however, that a potentially significant difference did exist in the ΔCt for wAlbA between third and fourth instars. Four instars had a significantly higher ΔCt than 3rd instars, indicating a higher load of wAlbA in third instars. It is unclear why wAlbA would decline in load during this period. However, *Wolbachia* load has been shown to be capable of decreasing in load with age (Calvetti, et al, 2016). This result does indicate the potential for differences in *Wolbachia* load during different stages of *Ae. albopictus* development. Further testing in mean ΔCt at different larval instars could confirm these differences.

The experiment also investigated the effects caused by extending the cold treatment, by leaving some samples in for an additional 7 days. Again this data was limited by small sample size, but the results of extending the duration were significant. In wAlbA, mean ΔCt was lower in the 14 day samples than in the 7 day samples, which that indicated wAlbA load was higher on average at day 14 than day 7. Previous work also established age specific trends in *Wolbachia* load for both strains in *Ae. albopictus* adults (Calvetti, et al, 2016). In males, wAlbA load seemed highest at emergence and to decline with age. In contrast, female wAlbA loads seemed to continuously increase with age. In the case of both sexes, it is possible that wAlbA load is increasing during larval development.

This result also fits into the broader literature on the growth of *Wolbachia*. The bacteria is known for its infection of host reproductive tissue, however it is also found in somatic tissue (Pietri, et al, 2016). There is evidence that *Wolbachia* relies on its host for many macronutrients (Ponton, et al, 2014; Molloy, et al, 2016) , and that its reproductive cycle may be related to host cell division (Ruang-Areerate, et al, 2008). It is unclear whether *Wolbachia* is mainly proliferating in reproductive tissue, or in all tissues. Thus it is unclear whether its load should be examined in the context of general larval development or just reproductive development. This experiment could not shed light on this subject, but it can provide data for future papers to aid in the investigation of host cell and *Wolbachia* replication interactions. This experiment hypothesized that both *Wolbachia* strains in *Ae. albopictus* rely on host cell division in general to increase in number, and that during mosquito development, when cell division is high, the bacteria are rapidly proliferating. The results cannot conclusively address that hypothesis, but they do indicate that further study may reveal differences in *Wolbachia* load based on host growth.

In conclusion, while the results of this project demonstrate that the treatment had no effect, they also support further investigation into *Wolbachia* load in *Ae. albopictus* larvae. All previous work indicated that wAlbB should be found at a higher density than wAlbA, and this experiment showed the same pattern. The major finding of both strains showing cold tolerance is compatible with the reality that *Ae. albopictus* regularly experiences these temperatures with no obvious reproductive phenotypic effects. There is less data to support the other conclusions of this paper, but they are an important starting point for further investigations. In particular, future experiments should study regional differences in *Wolbachia* load and differences between developmental stages. This project's findings may be applied in *Wolbachia* based mosquito

control. An understanding of cold tolerance in *Wolbachia* strains could allow for the development of strains with greater cold tolerance to be deployed against *Ae. albopictus* in the temperate regions it has invaded. Therefore, this research involving *Wolbachia* control strategies could potentially help to combat the risk *Ae. albopictus* poses as a major disease vector.

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