WOLBACHIA DENSITIES IN THE MOSQUITO Aedes aegypti

A Thesis in
Entomology

by

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ABSTRACT

The mosquito *Aedes aegypti* is a vector for viruses like dengue, chikungunya, and Zika. These arboviruses in most cases cause low mortality in humans but can lead to long-term complications. Dengue virus can also lead to severe forms resulting in death. Currently, there are no effective vaccines or drugs to combat these arboviruses. By 2050, it is expected that ~50% of the world will live in association with *Aedes* species vectors due to climate change and increasing urbanization. One critical tool to control arbovirus transmission in *Ae. aegypti* is the vertically inherited insect endosymbiont, *Wolbachia*. *Ae. aegypti* is not naturally infected with *Wolbachia*, but stably infected lines have been created by transinfection. *Wolbachia* causes two main traits of interest in mosquitoes; Cytoplasmic Incompatibility (CI) and viral blocking. CI manifests as embryonic death resulting from the mating of a *Wolbachia*-infected male with an uninfected female. *Wolbachia*-infected females do not suffer any such issue and so the symbiont tends to spread through populations. CI *Wolbachia*-infected males have been released into wild populations to crash native mosquito populations. Viral blocking is where *Wolbachia* limits the replication of dengue, chikungunya, and Zika viruses in the vector. Together with CI, this effect has led to the release of *Wolbachia*-infected females to replace native mosquito populations that then exhibit reduced virus transmission. In chapter 1, I reviewed both control strategies, how *Wolbachia*’s density affects CI and viral blocking strength, and the factors that affect *Wolbachia* density in insects. In chapter 2, I examined the relative densities of *Wolbachia* in somatic and reproductive tissues of *Ae. aegypti* and assessed whether densities are correlated across tissues in the same individual and across generations. In chapter 3, I attempted to create *Ae. aegypti* with stable low and high *Wolbachia* densities using artificial selection and inbreeding so that we could better study the basis of *Wolbachia*-mediated traits. Additionally, I assessed whether *Wolbachia* densities in mosquito legs could be used as sentinels for predicting densities in the remainder of
the insect body as a non-destructive means for accurately binning mosquitoes into high and low-density groups. Finally, in chapter 4, I discuss key findings in chapters 2 and 3, what they mean in our understanding of Wolbachia:insect associations, and what our results mean for field releases of Wolbachia-infected mosquitoes for biocontrol.
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Chapter 1

Introduction

1.1 Aedes aegypti

The geographic range of the mosquitoes *Aedes aegypti* and *Aedes albopictus* native to Africa and Asia, respectively, are expanding globally (Kraemer et al., 2019). Climate change and urbanization are both assisting the spread of these mosquitoes, and it is predicted that by 2050, 49% of the world’s population will live in association with these species (Kraemer et al., 2019). The two species transmit viruses to humans including dengue (DENV), chikungunya, Yellow Fever, and Zika. These arboviruses usually cause self-limiting disease characterized by fever, nausea, and joint pain, that can occasionally lead to long-term complications. Severe disease is also possible in the form of dengue hemorrhagic fever and for Zika in terms of microcephaly in the unborn. Dengue fever is by far the most common, with estimates of 50 million new infections each year globally (Beckham & Tyler, 2015). There are currently no licensed vaccines or effective antiviral drugs for these arboviral diseases (Merle et al., 2018). The primary means for limiting *Aedes* associated disease is through vector control, most commonly through the use of insecticides and the management of breeding sites. Despite widespread vector control programs, diseases like dengue fever continue to be on the rise (Bhatt et al., 2013), in part because of growing insecticide resistance (Wang et al., 2020).

1.2 Wolbachia

*Wolbachia* is an endosymbiotic bacterium that is found in ~50% of insect species (Hilgenboecker, Hammerstein, Schlattmann, Telschow, & Werren, 2008). It has been studied for
decades as a possible biocontrol agent because it causes cytoplasmic incompatibility (CI) and virus blocking. CI was first noticed in *Culex pipiens* mosquitoes by Marshall (1938) and later discovered to be caused by *Wolbachia* when the symbiont was discovered in the eggs of *C. pipiens* (Yen & Barr, 1971). CI manifests as embryonic death resulting from modifications to the sperm made by *Wolbachia* (J. H. Werren, 1997). CI only occurs in one particular cross between *Wolbachia*-infected/free mating pairs. Specifically, when a *Wolbachia* infected male mates with a *Wolbachia* free female, the offspring are non-viable. All other possible crosses lead to the production of normal offspring. The presence of *Wolbachia* in infected females, rescues the modification made by males.

As a consequence of CI, infected females have comparatively greater reproductive success than uninfected females. This advantage, in combination with the bacterium’s vertical inheritance from mother to egg, causes *Wolbachia* to spread quickly through populations (John H. Werren, Baldo, & Clark, 2008). In a variant of CI called bidirectional CI, embryonic death occurs when incompatible strains of *Wolbachia* encounter one another in matings. Offspring are rescued when similar strained males or females are mated. Bidirectional CI can drive speciation events, when populations of insects are infected with different *Wolbachia* strains other and become gene flow is cut off (Shropshire, Leigh, & Bordenstein, 2020).

*Wolbachia* prevents the replication of co-infecting viruses in insects in a trait known as *Wolbachia*-Mediated Pathogen Blocking (WMPB). WMPB was first noticed in the naturally *Wolbachia*-infected *Drosophila melanogaster*, when flies where infected with different insect specific viruses including; Drosophila C virus (DCV), cricket paralysis virus (CrPV) and Flock House virus (FHV) (Hedges, Brownlie, O’Neill, & Johnson, 2008). *Wolbachia* infected flies were able to live longer during viral infection than flies cleared of their symbionts with prior antibiotic
treatment (Hedges et al., 2008). Eventually it was determined that Wolbachia decreased the loads of these viruses in the host (Teixeira, Ferreira, & Ashburner, 2008).

Because of Wolbachia’s spreading and pathogen blocking abilities, researchers sought to transinfest the naturally Wolbachia free Ae. aegypti with strains donated from Drosophila (McMeniman et al., 2009; T. Walker et al., 2011a; Xi, Khoo, & Dobson, 2005). The process led to the creation of several stably infected lines with vertical inheritance of Wolbachia. Vector competence experiments in these mosquito lines, subsequently demonstrated that after consuming blood meals infected with both DENV and chikungunya that the mosquitoes had lower viral loads in their saliva and were therefore less likely to transmit virus (Bian, Xu, Lu, Xie, & Xi, 2010; Moreira et al., 2009). Eventually this same blocking ability was found for Zika virus (Dutra et al., 2016) as well as other pathogens, including the malaria parasite (Moreira et al., 2009), bacteria (Ye, Woolfit, Rancès, O’Neill, & McGraw, 2013), and filarial nematodes (Kambris, Cook, Phuc, & Sinkins, 2009a).

1.3 **Wolbachia based biocontrol**

Several biocontrol strategies have been developed to make use of CI and/or Wolbachia’s pathogen blocking ability. In the first approach, called Incompatible Insect Technique (IIT) aims reduce wild populations. With IIT, very large numbers of Wolbachia-infected males are released into populations that are naturally Wolbachia free, and due to the action of CI successful reproduction by the native females is prevented, crashing the population (Shropshire et al., 2020). Successful releases using IIT have occurred in Italy where the native population has started to decline and continued monitoring has found that ~30% of females captured were infertile (Caputo et al., 2020). A release in the US was able to reduce the population by ~90% compared to areas without releases (Crawford et al., 2020). In releases in China, populations were nearly all suppressed over a two-year period (Zheng et al., 2019). IIT is being supported by governments
often in collaboration with some private companies including MosquitoMate (Mains, Kelly, Dobson, Petrie, & Dobson, 2019) and Debug (debug.com).

A second strategy for Wolbachia-based biocontrol is called Population Replacement (PR) (Flores & O’Neill, 2018). Instead of reducing the population of mosquitoes it replaces the native population with Wolbachia-infected mosquitoes with a lower capacity to harbor arboviruses. PR uses both CI and Wolbachia’s pathogen blocking ability to accomplish this. Wolbachia infected females are released, producing offspring that carry Wolbachia, and in particular males that deliver the effect of CI. For Wolbachia to spread by the action of CI, large numbers of females, upwards of ~20% of the native population must be released (Axford, Ross, Yeap, Callahan, & Hoffmann, 2016) usually over a period of many weeks. Once Wolbachia goes to fixation in the population, because of Wolbachia-mediated pathogen blocking, the mosquito populations should be unable to transmit viruses. An original field release of Wolbachia in Cairns, Australia led to fixation in approximately 3-4 months (A. A. Hoffmann et al., 2011). Eight years after release, there have been no outbreaks of dengue in the region that normally saw an outbreak induced by traveler’s every year (Ryan et al., 2020). Other field releases have had similar levels of success.

In Malaysia, trial releases targeted communities characterized by densely populated high-rise buildings with nearly endemic or continuous transmission of dengue virus. After releases, people stopped using insecticides for vector control and reductions in the incidence of dengue fever were up to 40% (Nazni et al., 2019). The largest trial to date is currently underway in Yogyakarta, Indonesia. In four communities in Yogyakarta, Wolbachia has spread to fixation and remained stable for 3 years (Tantowijoyo et al., 2020). Research has found a 77% reduction of dengue fever in the release zone communities (Utarini et al., 2021).
1.4 Mechanisms of Wolbachia’s blocking ability

The mechanistic basis of Wolbachia’s blocking ability in mosquitoes is still not fully understood. The growing consensus is that the trait is multifaceted. Several processes in the mosquito that may be affected by Wolbachia and confer blocking include: immunity, RNA translation and replication, cellular stress, nutrient competition, and cell-cell adhesion. Several studies have demonstrated that Wolbachia upregulates immune gene expression in Ae. aegypti (Moreira et al., 2009; Rancès, Ye, Woolfit, McGraw, & O’Neill, 2012). This ‘immune priming’ may serve to increase basal immune protection allowing the mosquito to have greater control over dengue and other viruses it subsequently encounters during blood feeding. Interestingly, however, while there is virus blocking in the natively infected Drosophila melanogaster, this host exhibits very little upregulation of immune genes, suggesting that while immune priming may increase the strength of blocking in mosquitoes it is not likely the fundamental mechanism (Rancès et al., 2012). Another explanation for the basis of blocking is competition for cholesterol. Both Wolbachia and dengue virus are dependent on the host’s cholesterol for their replication and survival (Geoghegan et al., 2017; Heaton et al., 2010). A study in Wolbachia-infected Drosophila melanogaster found that flies reared on high cholesterol diets exhibited reduced pathogen blocking of viruses (Caragata et al., 2013). It is also suspected that viral RNA translation and/or replication may be prevented in the presence of Wolbachia (Rainey et al., 2016). Wolbachia is unable to synthesize certain amino acids that would help in its reproduction and growth. Instead, it relies on the host to do so (White et al., 2017; Wu et al., 2004) and uses transporters to take up the amino acids. Viruses use the host ribosome to carry out their own replication, which Wolbachia appears to alter (White et al., 2017). These changes may make the ribosome less hospitable for viruses (Lindsey, Bhattacharya, Newton, & Hardy, 2018). Wolbachia infection also induces oxidative stress that leads to the production of antimicrobial peptides in the host, which
were found to inhibit DENV proliferation (Pan et al., 2012). The induction of oxidative stress increases the Toll pathway that was found to decrease the spread of DENV in the host (Xi, Ramirez, & Dimopoulos, 2008). A recent study combining natural selection for blocking strength and GWAS approaches has demonstrated that SNP variation in cell-cell adhesion genes may underpin blocking (Ford et al., 2020). Cell adhesion may affect a virus’s ability to enter or move between cells which Wolbachia has been found to alter (Ford et al., 2019; Hughes et al., 2011).

1.5 Wolbachia densities

Regardless, of mechanism, there is some evidence that blocking strength correlates with Wolbachia density in both flies and mosquitoes (Amuzu & McGraw, 2016; Chouin-Carneiro et al., 2020; Chrostek et al., 2013b; Iturbe-Ormaetxe, Walker, & O’Neill, 2011; Rainey et al., 2016). If higher Wolbachia loads contribute to stronger blocking, keeping populations at high densities is crucial for successful biocontrol. If densities evolve to be lower in the vector, then blocking would decrease. This could occur from high fitness costs resulting from higher Wolbachia loads. Studies in Drosophila (Chrostek & Teixeira, 2015) and Ae. aegypti (Ant, Herd, Geoghegan, Hoffmann, & Sinkins, 2018) have found that higher densities result in higher fitness costs. Also, as fitness costs increase it is predicted that Wolbachia stops spreading in the population (T. Walker et al., 2011a). It is also possible that densities could decrease due to coevolution which has occurred in Drosophila in the laboratory (McGraw, Merritt, Droller, & O’Neill, 2002a).

Native hosts for Wolbachia tend to have lower loads and lower tissue distribution than artificially infected hosts (Bian, Zhou, Lu, & Xi, 2013; Miller, Ehrman, & Schneider, 2010; Osborne, Iturbe-Ormaetxe, Brownlie, O’Neill, & Johnson, 2012). The lower loads are thought to have resulted from a long history of coevolution. One concern for releasing Wolbachia is that through time densities will also evolve to be lower in wild populations. However, a study that examined Ae. aegypti from the field, 1 year post release, found that Wolbachia loads as well as viral blocking
were still the same as in parental line used for release (Frentiu et al., 2014). In the release zones in Malaysia, *Ae. aegypti* was found to have no decrease in *Wolbachia* loads 2 years post release (Ahmad et al., 2021).

Densities of *Wolbachia* in a host can vary by strain. A study by D. Albert Joubert et al., (2016) focused on two strains, wMel and wAlbB, from *Drosophila melanogaster* and *Ae. albopictus*, respectively. They infected *Ae. aegypti* with each strain singly and in combination called a superinfection (D. Albert Joubert et al., 2016). The density of the *Wolbachia* strain in the superinfected state was distinct from the single strains in the transinfected *Ae. aegypti* (D. Albert Joubert et al., 2016). In *Ae. albopictus*, the naturally superinfected Houston strain of wAlbA and wAlbB produces ovaries with higher levels of *Wolbachia* compared to that of wAlbB (Xi, Dean, Khoo, & Dobson, 2005). Even variations in the same strain can result in different densities in the mosquito. T. Walker et al., (2011a) found that the strain variants wMel and wMelPop-CLA can be found in the ovaries, salivary gland, Malpighian tubules, and the fat body in *Ae. aegypti*.

Densities of wMelPop-CLA are high in the Malpighian tubules and the fat body but low in those same tissues using wMel (T. Walker et al., 2011a). *Wolbachia*’s distribution in somatic tissue varies between species but it can be found in the head, muscles, midgut, salivary gland, fat body, and reproductive tissue, generally (Jervis, 2005; D. Albert Joubert et al., 2016; Pietri, DeBruhl, & Sullivan, 2016). Tissue distributions can be explained in part to differences in the *Wolbachia* strain as well as the particular host species. In some species such as the mosquito *Ae. albopictus* different strains were found to have *Wolbachia* present only in their reproductive tissue (Dobson et al., 1999). D. Albert Joubert et al., (2016) found that wMel is distributed throughout the egg chamber while wAlbB was primarily in the posterior when introduced into *Ae. aegypti*. The localization of wMel (Ferree et al., 2005) and wAlbB (Zouache et al., 2009) is similar in the
native host. Distribution of wAlbB was also found to be similar in oocytes in *Ae. aegypti* when compared to *Ae. albopictus* (Xi, Khoo, et al., 2005).

The density of Wolbachia across different insect associations differ (López-Madrigal & Duarte, 2020). Whether the variability in density is caused by the host or Wolbachia is not predictable. Wolbachia naturally occur in a large number of Drosophila species. When the Wolbachia strain, wMelPop, from *D. melanogaster* is transferred to *D. simulans*, densities are found at higher values than in the natural host (McGraw et al., 2002a). This suggests host specific control since densities vary between the two hosts and the tissues, they infect (McGraw et al., 2002a). If Wolbachia was in full control, then it would keep high densities in both hosts. When *D. serrata* is transinfected with Wolbachia from *D. simulans* lower densities of the symbiont were found in the recipient (Clancy & Hoffmann, 1997). Strain specific variation was also found in the parasitoid wasp, *Leptopilina heterotoma*, three different Wolbachia strains had different densities in the abdomen, head, and thorax (L. Mouton, Henri, Bouletreau, & Vavre, 2003). Two strains of *E. kuehniella* were infected with Wolbachia from the almond moth, *Cadra cautella*, one strain had more bacteria in the testis than the other (Ikeda, Ishikawa, & Sasaki, 2003). Buffalo flies, *Haematobia irritans exigua*, transinfected with three different strains of Wolbachia from *D. melanogaster* also resulted in different effects depending on the strain. Each strain had varying survival rates, emergence dates, egg laying amounts, and different levels of Wolbachia density in the head, thorax, midgut, fat body, and ovaries (Madhav et al., 2020). Two lines of the wasp, *Leptopilina heterotoma*, were infected with three different Wolbachia strains. Even though the same three strains were used each line differed in Wolbachia quantity when comparing the same strains (Laurence Mouton, Henri, Charif, Boulétreau, & Vavre, 2007). This result suggests that the host’s genotype plays a role in controlling Wolbachia’s density. Wolbachia’s density has also been found to be affect by the hosts autophagy mechanism. In male somatic cells of *D. melanogaster*, density is decreased due to autophagy (Deehan, Lin, Blum, Emili, & Frydman,
Cytoplasmic incompatibility (CI) strength has been previously linked to *Wolbachia* densities (López-Madrigal & Duarte, 2020). The almond moth, *Cadra cautella*, has two strains of *Wolbachia*. An experiment used these two strains and infected the flour moth, *Ephestia kuehniella*, with each (Sasaki, Kubo, & Ishikawa, 2002). They found that single infections of each variant gave different results, one resulted in male killing while the other had partial CI (Sasaki et al., 2002), despite no differences in densities of the two strains (Ikeda et al., 2003). In flies, naturally occurring *Wolbachia* in *D. melanogaster* do not result in CI (McGraw et al., 2002a). However, when the bacteria are transferred to the novel host, *D. simulans*, CI does occur (McGraw et al., 2002a). McGraw et al., (2002a) also found that *Wolbachia* densities were higher in the novel host compared to the natural. A metanalysis of different *Drosophila* hosts of *Wolbachia*, naturally infected and transinfected, found a relationship between infection and CI levels increase (Veneti et al., 2003). When sperm cysts are more infected CI levels increase and this was thought to explain the increase in novel hosts (Veneti et al., 2003). The host also has some control, however. *Wolbachia* transferred from *D. melanogaster* to *D. simulans* is found in sperm bundles (McGraw, Merritt, Droller, & O’Neill, 2001). The localization of the bacteria to these bundles is what facilitates CI expression, not found in the natural host (McGraw et al., 2001). Thus, *Wolbachia* is capable of inducing CI but in this case, there is host specific control on the locality of the bacterium. Sasaki & Ishikawa (1999) found that two different populations of *E. kuehniella* with closely related *Wolbachia* exhibited different levels of CI, one with 16% of eggs hatch and the other with 60%.

Temperature can also affect *Wolbachia*’s density. Laurence Mouton et al. (2007) infected two different lines of the wasp, *Leptopilina heterotoma*, with three different *Wolbachia* strains.
simultaneously. Wasps were reared at two different temperature; 20°C and 26°C, that resulted in varying *Wolbachia* densities. With increased temperature, *Wolbachia* loads rose in one wasp line but not in the other. The line with an increase was collected from a warmer region and may reveal evidence of past adaptation and host genotype control. Interestingly, when *Wolbachia* loads changed in the one wasp line, the relative proportions of the three strains remained the same. The results suggest that *Wolbachia* genotypes may have set replications rates that vary only by temperature. In mosquitoes, *Wolbachia* strains can have different temperature tolerances. For example, *w*Mel and *w*MelPop-CLA densities begin to decline in mosquitoes reared at temperatures in the range of 26-37°C, but the *w*AlbB strain density is maintained at these same temperatures (Ross et al., 2017).

*Wolbachia* densities between somatic and reproductive tissues can also vary. In *Culex pipiens*, *Wolbachia* is primarily found in reproductive tissue (Emerson & Glaser, 2017). When measuring the *Wolbachia* densities in the somatic vs reproductive tissue no correlation was found (Emerson & Glaser, 2017). This result suggest that in this species *Wolbachia* densities are controlled by different mechanisms between these two tissues. Ant et al. (2018) found that in *Ae. aegypti*, *Wolbachia* density is higher in ovaries compared to the midgut. This same result was found in 3 other *Wolbachia* strains in the same host (Ant & Sinkins, 2018). Similarly, when *Ae. albopictus* is infected with *Wolbachia* densities are higher in the ovaries than the midgut (Ant & Sinkins, 2018).

*Wolbachia* densities can also vary in a single host over time. For example, the host age can influence *Wolbachia* density. In flies, it has been found that density decreases for males as they get older (Reynolds & Hoffmann, 2002). However, in females, density increases with age (Kaur, Martinez, Rota-Stabelli, Jiggins, & Miller, 2020). These same trends have been seen in mosquitoes (Pablo Tortosa et al., 2010). An exception to this trend has been found in *C. pipiens*, were densities in the testes increased with age (Duron, Fort, & Weill, 2007). In flies (Bressac &
Rousset, 1993), wasps (Breeuwer & Werren, 1993), and moths (Ikeda et al., 2003) the opposite occurred.

Coevolution is when two organisms affected each other’s evolution. *Wolbachia* could coevolve with its host. A possibility is that coevolution could lower densities in the host. When *Wolbachia* was transferred from *D. melanogaster* to mosquito cells back to the native host densities were at lower quantities (McMeniman et al., 2008). As expected, this resulted in weaker CI (McMeniman et al., 2008). This result shows that coevolution is possible and that densities could decrease through generations leading to weaker CI expression.

### 1.6 Wolbachia from embryo to adult

*Wolbachia* tissue distributions in the early embryo define later tissue patterns in the adult. In *Drosophila*, most of the bacteria are found near the cortical regions of the egg, 90% (Boyle, O’Neill, Robertson, & Karr, 1993). During early development of the egg, *Drosophila* nuclei begin to proliferate throughout the cytoplasm. *Wolbachia* is found to attach to the nuclei and thus uses this relationship to hitchhike through the cytoplasm (Kose & Karr, 1995). As the nuclei move to the periphery of the egg, so does the bacteria (Kose & Karr, 1995). Throughout this process *Wolbachia* density does not change (Kose & Karr, 1995). However, density in the egg can vary based on the host. For example, in *D. melanogaster*, *Wolbachia* density in the egg cytoplasm was lower compared to *D. simulans* (Callaini, Riparbelli, & Dallai, 1994). The movement of *Wolbachia* to the polar sides of the egg is what facilitates vertical transmission since these sections become germ cells (Ferree et al., 2005). However, the dispersion of *Wolbachia* throughout the cytoplasm and its role in development is still unclear. A possibility is that the dispersal throughout the cytoplasm helps to dictate what tissues will have *Wolbachia* (Pietri et al., 2016). In some strains of *Wolbachia*, the bacteria moves towards the posterior of the egg rather than the typical anterior site (Veneti, Clark, Karr, Savakis, & Bourtzis, 2004). While another
strain keeps high densities throughout the cytoplasm (Serbus & Sullivan, 2007). These differences cause variable Wolbachia densities in somatic tissues. It is also unclear as to what mechanism triggers Wolbachia to increase its density once the host becomes an adult. wMelpop is a Wolbachia strain that reaches high densities in the host resulting in death. Yet, during embryogenesis its density is low (Min & Benzer, 1997). It is not until it reaches the adult stage the bacteria begin to proliferate (Min & Benzer, 1997).
1.7 References


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Chapter 2

Cross tissue and generation predictability of Wolbachia densities in the mosquito Aedes aegypti

2.1 Abstract

Background: The insect endosymbiont, Wolbachia, is being deployed in field populations of the mosquito Aedes aegypti for biological control, as the microbe prevents the replication of the human disease-causing viruses inside the vector including dengue, Zika, and chikungunya. Relative Wolbachia densities may in part predict the strength of this ‘viral blocking’ effect. Additionally, Wolbachia densities may affect the strength of the reproductive manipulations it induces including Cytoplasmic Incompatibility, maternal inheritance rates, or induced fitness effects in the insect host. High rates of CI and maternal inheritance, and low rates of fitness effects are also key for Wolbachia to spread successfully through vector populations be used successfully for biocontrol. The factors that control Wolbachia densities are not completely understood.

Methods: Here we use quantitative PCR-based methods to estimate relative density of the Wolbachia wAlbB strain in both the somatic and reproductive tissues of adult male and female mosquitoes, as well as in eggs. Using correlation, we assess whether densities in one tissue predict those in others within the same individual, but also across generations.

Results: We show little relationship between the relative Wolbachia densities of different tissues in the same host. We also show very little relationship between Wolbachia densities in parents and offspring, both in the same and different tissues. The only exception was with ovary-egg relationships, where there was a strong positive association. Relative Wolbachia densities in reproductive tissues were always greater than those in the somatic tissues. Additionally, we saw
that densities were consistent in females over their lifetime regardless of tissue, whereas they were generally higher and more variable in males, particularly in the testes.

**Conclusions:** Our results indicate that either stochastic processes or local tissue-based physiologies are more likely dictating *Wolbachia* densities in *Ae. aegypti* individuals, rather than shared embryonic environments or heritable genetic effects of the mosquito genome. These findings have implications for understanding how relative *Wolbachia* densities may evolve or be maintained over the long term in *Ae. aegypti*.

**Keywords:** *Wolbachia*, density, *Aedes aegypti*, mosquito, symbiont

**Abbreviations:** CI: cytoplasmic incompatibility; WMPB: *Wolbachia*-Mediated Pathogen Blocking; H+T: head and thorax; CP: Crossing Point
2.2 Introduction

The global geographic range of the mosquito vector, *Aedes aegypti* is expanding (Kraemer et al., 2019). This mosquito transmits the human disease-causing viruses dengue, Zika, chikungunya, and yellow fever (Souza-Neto, Powell, & Bonizzoni, 2019). Because there are no commercially available vaccines for these viruses, vector control remains the primary mechanism for limiting human disease (Merle et al., 2018). *Wolbachia pipiensis* is an endosymbiotic bacterium found in ~50% of all known insect species (Hilgenboecker et al., 2008). The symbiont induces two phenotypes that complement each for use in vector-borne disease control. First, it causes Cytoplasmic Incompatibility (CI), whereby offspring from crosses between *Wolbachia*-infected males and *Wolbachia*-free females are non-viable. The result is that *Wolbachia*-infected females have greater relative reproductive success and because *Wolbachia* is maternally inherited vertically via the egg, the symbiont spreads through populations (John H. Werren et al., 2008). Second, *Wolbachia* has also been found to limit the replication of co-infecting viruses in many insects including *Ae. aegypti* (Bian et al., 2010; Dutra et al., 2016; Moreira et al., 2009), in a trait known as *Wolbachia*-Mediated Pathogen Blocking (WMPB). *Ae. aegypti* in the wild are naturally free of *Wolbachia*, but laboratory populations have been artificially and stably infected with the symbiont (Ahantarig, Trinachartvanit, & Kittayapong, 2008; McMeniman et al., 2009; T. Walker et al., 2011a; Xi, Dean, et al., 2005; Xi, Khoo, et al., 2005).

In the field, *Wolbachia* is being evaluated for vector-borne disease control via two means; population suppression and population replacement (Shropshire et al., 2020). Suppression involves releasing *Wolbachia* infected males only, to prevent the successful reproduction of wild *Wolbachia*-free females, leading to population crashes. In replacement strategies, *Wolbachia*-infected females are released in large numbers to seed the next generation with *Wolbachia* infected offspring. The daughters become part of the maternal transmission cycle and the sons assist with *Wolbachia* spread.
via the action of CI. The result is a population with high rates of *Wolbachia* infection and a poor ability to transmit viruses (A. A. Hoffmann et al., 2011; Nazni et al., 2019). Both strategies are showing high rates of efficacy in the field (Caputo et al., 2020; Tantowijoyo et al., 2020). The continued success of these approaches relies on the ongoing strength of CI and WMPB expression. *Wolbachia* densities appear to predict both strengths of CI and WMPB (Amuzu & McGraw, 2016; Calvitti, Marini, Desiderio, Puggioli, & Moretti, 2015; Chrostek et al., 2013a; Clancy & Hoffmann, 1998; Martinez et al., 2014; Noda, Koizumi, Zhang, & Deng, 2001; Veneti et al., 2003).

The factors that control *Wolbachia* densities are not fully understood (López-Madrigal & Duarte, 2020), but appear to involve both host and symbiont genetics (Chrostek & Teixeira, 2018; Laurence Mouton et al., 2007) and a range of environmental effects including temperature and host nutrition (Duron et al., 2007; Kaur et al., 2020; Laurence Mouton et al., 2007; Serbus et al., 2015). Even within an individual insect, *Wolbachia* densities can vary highly between tissues. Reproductive tissues often exhibit higher densities, although in Drosophila this can depend on the *Wolbachia* strain (Kaur et al., 2020; Osborne et al., 2012). Higher reproductive tissue densities have been noted in the mosquitoes *Ae. aegypti* (Ant et al., 2018), *Aedes albopictus* (Ant & Sinkins, 2018), and *Culex pipiens* (Emerson & Glaser, 2017). The relative contributions of *Wolbachia/vector genetics vs. environmental effects in determining *Wolbachia* tissue densities is not known. Studying the heritability of *Wolbachia* densities in female lineages is challenging, given that the shared environment of the ovaries/eggs confounds any estimates of contributions from genetic factors. In this study, we sought to understand the relationship between *Wolbachia* densities in somatic and reproductive tissues within individuals and across generations in the artificially wAlbB strain transinfected *Ae. aegypti* using quantitative PCR-based methods. An understanding of the relative role of genes and environment in determining *Wolbachia* densities may have consequences for the deployment and use of *Wolbachia*-based biocontrol where key phenotypes depend on density.
2.3 Methods

2.3.1 *Ae. aegypti* rearing

We used a population of *Ae. aegypti* infected with the wAlbB (Xi, Khoo, et al., 2005) strain of *Wolbachia* donated by Zhiyong Xi (Michigan State). Prior to experimentation, we outcrossed females of wAlbB to *Wolbachia*-free male mosquitoes recently obtained from Monterrey, Mexico (Pablo Manrique-Saide, Universidad Autónoma de Yucatán) for three generations to increase genetic diversity. Eggs were hatched in 40 × 30 × 8 cm plastic trays with 2 liters of distilled deoxygenated water. Larvae were maintained at a density of ~250 per tray and fed Tetramin fish food (Melle, Germany) *ad libitum*. Pupae were collected in plastic cups and placed in 45 cm square breeding cages (BioQuip) in populations of ~300 individuals. Adult mosquitoes were fed 10% sucrose solution *ad libitum*. Mosquitoes were blood-fed at 9-11 days of age with human blood using a Hemotek feeder (Hemotek Ltd) warmed to 37°C. For experiments that involved egg collection, 3 days post blood feeding, females were individually placed in 70 ml oviposition cups containing moist filter paper and access to 10% sucrose.

2.3.2 Experimental design

We measured *Wolbachia* densities in three ways; between somatic and reproductive tissues in the same individuals, across generations in tissues of parents and offspring, and in tissues of the same individuals across their lifespan (Figure 2.1). Our aim was to examine whether relative tissue densities correlated in these different contexts. For the within individual mosquito comparisons (Figure 2.1A), we set up two experiments. First, we setup 206 mated blood fed individuals as isofemales at 9-11 days of age. We dissected the ovaries and the carcass (remaining tissues) at 15-17 days of age, or ~6 days post-feed. Second, we set up two replicate groups of 18 blood feed isofemales so that in addition to ovaries and the carcass we could also correlate egg densities within
individuals. Eggs were extracted for DNA in groups of 10 per isofemale, after pilot experiments revealed this was the minimum pool size needed for consistent *Wolbachia* density estimation. For across generation comparisons (Figure 2.1B), we also set up blood fed isofemales (~200) as above. After collecting their eggs, we dissected ovaries and carcass in the mothers (P$_1$). We hatched the eggs laid by each isofemale separately, and then reared, fed, and dissected these F$_1$ families as described for P$_1$. *Wolbachia* densities were estimated from tissues extracted individually from 2-3 F$_1$ daughters per P$_1$ mother and then averaged. We also wanted to correlate relative *Wolbachia* density in the eggs, to the ovaries and the carcass across a generation. We, therefore, set up a new colony, collected eggs from P$_1$ mothers, and extracted pools of 10 eggs per female. We hatched the remaining eggs to create 20 cages of ~50 individuals. F$_1$ families were dissected identically to F$_1$ families above. Relative *Wolbachia* densities were estimated in tissues extracted from 3-8 F$_1$ daughters per P$_1$ mother and then averaged. For the experiment examining relative *Wolbachia* densities in tissue over the mosquito’s lifespan (Figure 2.1C) we set up a cage of ~250 individuals. We dissected males and unblood-fed females for their reproductive tissues, abdomen, head, and thorax at 5, 10, and 15 days of adulthood.
Figure 2.1: Tissues collected for each experiment. A. Within individual tissue correlation. B. Parent-offspring correlation. C. Tissue comparisons over the mosquito’s lifespan.
2.3.3 Dissections and DNA extraction

Females were anesthetized via CO$_2$ and dissected in 1x phosphate buffered saline (PBS). Tissues were collected and placed in a 2 ml tube with 50 μl of PBS and a 3-mm glass bead. Dissected tissues were stored at -80°C until processing. Similarly, eggs were collected in groups of 10. To extract DNA, tubes were filled with 50 μl of extraction buffer (10 mM Tris buffer, 1 mM EDTA, 50 mM NaCl, and proteinase K). Samples were homogenized with a bead ruptor (OMNI International) for 90 sec., centrifuged at 2000g for 2 mins and then incubated at 56°C for 5 min and at 98°C for 5 min. A final centrifugation step was performed at 2000g for 2 mins to pellet any remaining mosquito tissue. Samples were diluted 1:10 using DNAse/RNAse free water prior to quantification.

2.3.4 Wolbachia quantification

While there are methods for estimating absolute numbers of Wolbachia (Xu et al., 2013) we have chosen to measure relative abundance. This method is more appropriate when you are interested in capturing Wolbachia density in a given tissue and when comparing across tissues where the size of tissue (# cells) will vary. Densities rather than absolute numbers may be more revealing when attempting to capture the ‘concentration’ of Wolbachia, which may affect the strength of Wolbachia-mediated phenotypes (Amuzu & McGraw, 2016; Calvitti et al., 2015; Chrostek et al., 2013a; Clancy & Hoffmann, 1998; Martinez et al., 2014; Noda et al., 2001; Veneti et al., 2003). The relative method of estimation could be misleading, however, if ploidy numbers differ across tissues (Xu et al., 2013). Since there is little study of ploidy by cell or tissue type in mosquitoes, in addition to providing Wolbachia gene to host gene ratios, we also provide our raw Crossing Point (CP) values for the mosquito control gene in the supplemental files to demonstrate their uniformity. Average rps17 values for all tissues vary less than 2-fold across the samples (see specific figures/appendix file pairs in results below) suggesting low variability.
densities were quantified through qPCR using Livak’s method (Livak & Schmittgen, 2001) and a set of previously published primers for wAlbB ankyrin repeat domain gene (Axford et al., 2016) and the mosquito ribosomal subunit protein S17 (RpS17) (Ford et al., 2019). Wolbachia primers were as follows: wAlbB_F (5’- CTTACCTCCTGCACAACAA) and wAlbB_R (5’- GGATTGTCCAGTGGCCTTA) (Axford et al., 2016), and for mosquito: RPS17_F (5’- TCCGTGGTATCTCCATCAAGCT) and RPS17_R (5’- CACTTCCGGCACGTagTTGTC) (Ford et al., 2019). Quantitative PCR was carried out on a LightCycler 480 (Roche), using the equation $2^{- \frac{wAlbB}{RPS17}}$ (Livak & Schmittgen, 2001), with a total volume of 10μL per reaction, each containing: 5μL of 2x PerfeCTa SYBR Green SuperMix (Quantabio), 0.2μL of each forward and reverse primers (10μM), 2.6μL of nuclease-free water and 8uL of template DNA. The qPCR temperature profile was as follows: denaturation at 95°C for 5 min, 45 cycles of 95°C for 10 sec, 60°C for 15 sec and extension at 72°C for 10 sec, followed by a melt curve analysis.

2.3.5 Statistical analysis

All statistical analyses for the ‘within individual’ (Figure 2.1A) and ‘across generation’ (Figure 2.1B) experiments were performed in GraphPad Prism version 9.1.0 for Windows (GraphPad Software). Data were checked for normality before performing analysis and logarithmically transformed when necessary. All relative densities when depicted in scatter plots were plotted on a log axis. Fitted regression lines, although linear, can therefore appear curved. Paired t-tests were performed when comparing ovaries and carcass. A one-way ANOVA was used to compare relative Wolbachia densities with ‘Tissue’ as a fixed effect. Tukey’s post hoc comparisons were used to individually compare the densities in the ovary, carcass, and eggs. Analysis of Wolbachia densities in the ‘across mosquito lifespan’ experiment (Figure 2.1C) was performed in JMP 16.0.0 (SAS Institute Inc). A three-way ANOVA was used to compare the factors sex, time, and tissue, followed by selected post hoc comparisons.
2.4 Results

2.4.1 Within individual relative *Wolbachia* tissue density comparisons

We measured relative *Wolbachia* density between ovaries and carcass ~6 days post blood feeding (15-17 days of adulthood) in the same individuals to see whether tissue densities were correlated with one another (Figure 2.1A). We found that ovary densities were significantly greater (2-fold higher) than carcass densities (P<0.0001) (Figure 2.2/Raw CP Appendix). Relative densities in the ovaries ranged from ~2 to ~491 and in the carcass from ~0 to ~230, also reflecting a wider variation in density in the reproductive tissue. We found no correlation between ovaries and carcass relative densities (P=0.13) (Figure 2.3). We then measured relative *Wolbachia* densities between ovaries, carcass, and eggs in the same individuals to assess whether there were correlations (Figure 2.1A). To accomplish this, we set up two replicate groups of 18 individuals. We found that ovary densities were 5-fold (Figure 2.4/ Raw CP Appendix) and 10-fold (Appendix) higher than densities in eggs produced by the same individuals (Tukey’s multiple comparison test: P=<0.0001). Carcass densities were also 2-fold (Figure 2.4) (Tukey’s multiple comparison test: P=<0.0001) and 4-fold (Appendix) (Tukey’s multiple comparison test: P=<0.0001) higher than egg densities. In one group, the ovary densities were 2-fold higher than carcass (Figure 2.4) (Tukey’s multiple comparison test: P=<0.0001), but there was no significant difference in the second replicate (Appendix) (Tukey’s multiple comparison test: P=0.33). Both groups exhibited the same trend of higher relative *Wolbachia* density in eggs correlating with higher ovary densities for replicate 1 (P=0.043) (Figure 2.5A) and replicate 2 (P=0.0062) (Appendix). In both replicate 1 (P=0.91) (Figure 2.5B) and replicate 2 (P=0.13) (Appendix) there was no correlation between egg and carcass density. Overall, egg densities were far less variable than ovary or carcass densities.
Figure 2.2: Relative Wolbachia densities (ankyrin repeat domain to rps17) in the ovaries and the carcass of Ae. aegypti in the same generation of mothers (P1). n=206, P<0.0001 (paired t-test). Bars indicate tissue means ± SE; ****P ≤ 0.0001.

Figure 2.3: Relationship between relative Wolbachia densities (ankyrin repeat domain to rps17) in the ovaries and the carcass of Ae. aegypti in the same generation of mothers (P1). n=206.
Figure 2.4: Relative Wolbachia densities (ankyrin repeat domain to rps17) in the ovaries, carcass, and eggs of *Ae. aegypti*. One-way ANOVA $P<0.0001$; post-hoc Tukey’s test: ovaries versus eggs: $P<0.0001$; carcass versus eggs: $P<0.0001$; ovaries versus carcass: $P<0.0001$. n=18 individuals. Bars indicate tissue means ± SE; ****$P \leq 0.0001$

Figure 2.5: Relative Wolbachia densities (ankyrin repeat domain to rps17) in the ovaries, carcass, and eggs of replicate group 1 of *Ae. aegypti*. A. *Wolbachia* densities in the eggs versus the ovaries of *Ae. aegypti* in replicate group 1. B. *Wolbachia* densities in the eggs versus the carcass of *Ae. aegypti* in replicate group 1. n=18 individuals.
2.4.2 Across generation relative Wolbachia tissue density comparisons

We then examined whether tissue densities in female offspring could be predicted based on densities in the female parent (Figure 2.1B). We saw no relationship between P1 ovaries and F1 carcass densities (P=0.25) (Figure 2.6A/ Raw CP Appendix) or between P1 carcass and F1 ovary densities (P=0.97) (Figure 2.6B). Similarly, we found no correlation between P1 and F1 ovary densities (P=0.58) (Figure 2.6C), nor between carcass densities (P=0.33) (Figure 2.6D). We did find a negative correlation between densities in P1 eggs and F1 ovaries (P=0.0005) (Figure 2.7A/ Raw CP Appendix). We found no correlation between P1 eggs and F1 carcass densities (P=0.51) (Figure 2.7B).
Figure 2.6: Relative Wolbachia densities (ankyrin repeat domain to rps17) in the tissues of mothers (P₁) versus daughters (F₁).

A. Wolbachia densities in the ovaries of P₁ versus the carcass of F₁ in Ae. aegypti. B. Wolbachia densities in the carcass of P₁ versus the ovaries of F₁ in Ae. aegypti. C. Wolbachia densities in the ovaries of P₁ versus the ovaries of F₁ in Ae. aegypti. D. Wolbachia densities in the carcass of P₁ versus the carcass of daughters F₁ in Ae. aegypti. Each data point represents the average of 2-3 individuals. For A and B n=31, while for C and D n=30 data points.

Figure 2.7: Relative Wolbachia densities (ankyrin repeat domain to rps17) in the eggs of mothers (P₁) versus tissues of daughters (F₁). A. Wolbachia densities in the eggs versus the ovaries of Ae. aegypti. B. Wolbachia densities in the eggs versus the carcass of Ae. aegypti. Each data point represents the average of 3-8 individuals. n=20 data points.
2.4.3 Across lifetime relative Wolbachia tissue density comparisons

To assess whether symbiont densities change with time, relative Wolbachia density was measured in the reproductive tissue, the abdomen, and a combination of the head and thorax (H+T) of male and non-bloodfed female mosquitoes at 5, 10, and 15 days of age. A three-way ANOVA between sex, time, and tissue resulted in sex (P<0.001), tissue (P<0.001) being significant, and the interaction between the two factors (P=0.0004) (Figure 2.8). The H+T (Tukey’s multiple comparison test: P=0.0098) and abdomen (Tukey’s multiple comparison test: P=0.0007) densities remained largely stable over the lifetime in both males and females. On average across all days, reproductive tissue densities in males were more variable through time and higher than female reproductive tissue (Tukey’s multiple comparison test: P<0.0001).

Figure 2.8: Relative Wolbachia densities (ankyrin repeat domain to rps17) across Ae. aegypti lifetime. A. Relationship between Wolbachia densities in the ovaries, abdomen, and head + thorax (H+T) of Ae. aegypti females at different time points. n=14-15 individuals. (Tissue=P<0.0001; Time=P=0.50; Time x Tissue=P=0.60). B. Relationship between Wolbachia densities in the testes, abdomen, and head + thorax (H+T) of Ae. aegypti males at different time points. n=12-15 individuals. Only sex (P<0.001) and tissue were significant (P<0.001).

2.5 Discussion

Wolbachia’s distribution in somatic tissues varies between species but the symbiont can be found in the head, muscles, midgut, salivary gland, fat body, as well as reproductive tissues
(Jervis, 2005; D. Albert Joubert et al., 2016; Pietri et al., 2016). In keeping with previous findings for *Drosophila* (Martinez et al., 2015), and *Aedes* (Zouache et al., 2009), our relative *Wolbachia* densities were higher in reproductive tissues compared to somatic. Additionally, we show in our study, that densities in the ovaries and carcass, and the carcass and eggs, in the same individual are largely independent of one another. This disconnect can be first be attributed to the distribution of *Wolbachia* in the early embryo. In *Drosophila*, *Wolbachia* attach to the proliferating nuclei and use this relationship to hitchhike through the oocyte (Kose & Karr, 1995). This places some *Wolbachia* in the periphery of the egg, where reproductive cells are formed (Serbus & Sullivan, 2007). However, a high fraction of the bacteria remain throughout the oocyte (Veneti et al., 2004). It is believed that this early embryonic distribution is what dictates which somatic tissues will have *Wolbachia* and in part, their relative *Wolbachia* densities (Pietri et al., 2016). Little is known about *Wolbachia*'s life cycle during the embryonic development of mosquitoes. However, *Drosophila* and *Aedes* have very similar embryogenesis stages that differentiate by timing (Clemons et al., 2010).

In addition, local tissue-specific factors could also be driving differences in relative densities including, immunity, physical niches, or access to nutritional resources. For instance, the insect immune response can positively or negatively affect *Wolbachia* densities, in a tissue-specific manner. Autophagy is a pathway that involves the degradation of “unwanted” components such as pathogenic bacteria. In the somatic cells of male *Drosophila*, the autophagic response reduces *Wolbachia* density, but in germ cells of females, the opposite occurs (Deehan et al., 2021). In multiple studies, infection with another bacterium (Ye et al., 2013) or virus (P. Tortosa, Courtiol, Moutailler, Failloux, & Weill, 2008; Ware-Gilmore et al., 2021) that triggers the innate immune Toll and IMD pathways, appear to also cause reductions in *Wolbachia* density. We know from transcriptional studies that the activity of these pathways varies across mosquito tissues including the midgut, carcass, and salivary gland when induced by infection (Sigle &
McGraw, 2019), but their basal expression can also vary as per an examination of the control data for these same studies (Bonizzoni et al., 2012; Sim, Ramirez, & Dimopoulos, 2012). One somatic tissue with a very high Wolbachia relative density in *Ae. aegypti* is the Malpighian Tubules (Albert Joubert et al., 2016). As the main site of nitrogen secretion, these tissues may especially facilitate Wolbachia growth, given that the symbiont primarily consumes host nucleotides (Wu et al., 2004), a big source of nitrogen. This same study also revealed pockets of cells, within particular tissues like the midgut epithelia, thoracic ganglia, and the salivary glands, that exhibit higher relative densities than the surrounding tissues (D. Albert Joubert et al., 2016). The reason for either Wolbachia’s tropism to particular cell types or greater replicative success in these sub-tissue level environments is unknown.

Our results did show a correlation between relative ovary densities and eggs produced from the same individual, which is expected given the egg’s origin and Wolbachia’s vertical inheritance (Horne-Badovinac, 2014). Wolbachia’s ongoing transmission success depends on its density in the ovaries. A range of studies, from Drosophila, shed light on the interactions between Wolbachia and the female germline that may also be relevant for mosquitoes. For example, Wolbachia increases the production of fly proteins in the ovaries that protect the germline from iron toxicity, oxidative stress, and increase the rate of stem-cell division (Christensen et al., 2016). The increased prevalence of these proteins may aid Wolbachia’s own proliferation and ensure transmission (Christensen et al., 2016). Also, Wolbachia has a tropism for the ovarian stem-cell niche. Once there Wolbachia increases germline stem-cell division and stops programmed cell death resulting in higher egg production (Fast et al., 2011). Additionally, Wolbachia’s tropism to ovarian stem-cell niches has been found to increase bacterial density in the germline (Toomey, Panaram, Fast, Beatty, & Frydman, 2013). Therefore, Wolbachia ensures vertical inheritance by increasing egg production and its density in the germline.
In contrast to the ovary/egg relationship, we did not see predictability of relative \textit{Wolbachia} densities across generations for other tissues. As we compare across generations, we consider the relative contributions of genes and environment toward the determination of density vs. stochastic processes. Temperature and diet have been shown to affect relative \textit{Wolbachia} densities (Madhav et al., 2020; L. Mouton et al., 2003; Laurence Mouton et al., 2007; Serbus et al., 2015). However, a previous study has shown that \textit{wAlbB} remains at a constant density between 26-37°C (Ross et al., 2017) and under laboratory rearing our temperatures should be largely constant. Similarly, given the low-density rearing of larvae and \textit{ad libitum} food delivery in both juveniles and adults, nutrition should have minimal impact on densities in our design. Host genetic factors cause varying \textit{Wolbachia} density in arthropods (Kaur et al., 2020; López-Madrigal & Duarte, 2020). Our poor cross-generation predictability, however, is more in keeping with a recent paper demonstrating that genetic drift is a more likely cause for dictating density (Bénard et al., 2021). The cause of this drift can likely be attributed to the uneven passage of \textit{Wolbachia} from mother to egg causing siblings to have varying densities (Bénard et al., 2021).

One caveat or our study is that we focused only on the \textit{wAlbB} \textit{Wolbachia} strain. Future studies may wish to assess the generality of our findings for other strains in \textit{Ae. aegypti}. While a previous study in \textit{Ae. albopictus}, (Ahantarig et al., 2008) also showed no relationship between mother to offspring densities for both \textit{wAlbA} and \textit{wAlbB} strains, the more distantly related \textit{wMel} strain could differ. A recent study in flies showed very large differences in relative \textit{Wolbachia} tissue densities depending on the \textit{Wolbachia} strain:host species pairing (Kaur et al., 2020). Our findings have potential implications for \textit{Wolbachia}-based biocontrol in the field. In the longer term, any directional selection on \textit{Wolbachia} densities in the ovaries, may not have a similar predictable effect on the body wide densities, as well as the converse. This is important as the former is thought to maintain transmission and CI expression (Layton, On, Perlmutter, Bordenstein, & Shropshire, 2019; Thomas Walker et al., 2021), whereas the latter is likely to
control pathogen blocking (Terradas, Allen, Chenoweth, & McGraw, 2017). Infection on both types of tissues may have direct impacts on host fitness (Ritchie, Townsend, Paton, Callahan, & Hoffmann, 2015). This also means that artificial selection to create mosquito lines with higher or lower Wolbachia in their various tissues is unlikely to be effective. Identifying Wolbachia strains for transinfection that exhibit differences in density either singly or when in superinfection with other strains (Ant et al., 2018; Dutton & Sinkins, 2004; D. Albert Joubert et al., 2016) may offer the most effective means for generating strain density diversity (Dirk Albert Joubert & O’Neill, 2017).

2.6 Conclusions

This study suggests that in Ae. aegypti that local tissue-based environments including factors (like nutrition, cellular niches, or immunity), initial differential distributions of Wolbachia in the dividing embryo, or stochastic factors like partitioning of density-associated Wolbachia genotypes into the embryo are likely more powerful than shared embryonic environments and shared inheritance through a female genetic line in determining relative symbiont densities. The relatively narrow variation in egg densities, ultimately resulting in highly variable densities in adult tissues, is also in keeping with this hypothesis. Future comparative studies may seek to understand how distinct tissue and cellular niches either promote or limit relative Wolbachia densities. The growing use of single cell RNAseq approaches in insects (Li, 2021) may assist with these comparisons. At the level of the vector, the effect of environmental conditions, more representative of natural field settings (Foo, Hoffmann, & Ross, 2019), may introduce further variability in densities, Wolbachia inheritance, and the expression of Wolbachia-induced traits key for biocontrol strategies.
2.7 Acknowledgments

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2.8 References


Chapter 3

Attempts to create *Aedes aegypti* lines with distinct and stable relative *Wolbachia* densities using artificial selection and inbreeding

3.1 Abstract

**Background:** *Wolbachia* is an insect endosymbiont being used for biocontrol in the mosquito *Aedes aegypti* due to the phenotypes it induces, Cytoplasmic Incompatibility (CI), and viral blocking. *Wolbachia* have been found to limit replication of dengue, chikungunya, and Zika virus in *Ae. aegypti*. The mechanism(s) of blocking is not fully understood, but we know that blocking strength and CI are positively correlated to *Wolbachia* densities in the host. We are unable to genetically modify *Wolbachia*, making it challenging to study the basis of *Wolbachia*-mediated traits. If we could create *Ae. aegypti* lines with stable and distinct (high/low) relative symbiont densities for a single *Wolbachia* strain and mosquito species association, we could more powerfully study traits like CI and viral blocking.

**Methods:** Here we used the tools of artificial selection and inbreeding in an attempt to create lines of *Ae. aegypti* with heritable high and low *Wolbachia* densities. We also explore using relative *Wolbachia* densities in mosquito legs to accurately bin the remaining mosquito carcass into high or low-density categories for use in non-DNA -omics based analysis.

**Results:** We were unable to significantly shift relative *Wolbachia* densities either through artificial selection or inbreeding, with lines instead showing a repeated pattern of cyclical change. We do show a relationship between relative *Wolbachia* densities in the legs and carcass that could be used to accurately sort carcass collections into distinct high and low density categories.

**Conclusions:** Our results suggest that either the host, the symbiont, or some interaction between the two partners are likely modulating relative *Wolbachia* densities over a narrow range. This finding suggests that artificial selection or inbreeding will not be effective tools in attempts
to generate *Ae. aegypti* with different relative densities for potential field deployment. It also suggests that *Wolbachia* densities are highly regulated in this association and therefore more likely to remain stable in the field. Regardless, we have demonstrated an experimental approach for accurate within generation characterization of relative densities that could advance our ability to functionally study the basis of *Wolbachia*-mediated traits that correlate with density.

**Keywords:** *Wolbachia*, mosquitoes, density, artificial selection
3.2 Introduction

*Aedes aegypti*’s global geographic range is expanding (Walker et al., 2011a). This mosquito transmits human disease-causing viruses including dengue, chikungunya, Zika, and yellow fever (Souza-Neto, Powell, & Bonizzoni, 2019). There are currently no effective vaccines or antiviral drugs for these arboviruses (Merle et al., 2018). Instead, we rely on vector control to suppress arboviral spread worldwide. *Wolbachia pipiens* is a bacterial endosymbiont found in ~50% of insect species (Hilgenboecker, Hammerstein, Schlattmann, Telschow, & Werren, 2008). *Wolbachia* induces two traits in mosquitoes that are the basis of its use for vector control (Flores & O’Neill, 2018), Cytoplasmic Incompatibility (CI) and *Wolbachia*-Mediated Pathogen Blocking (WMPB). CI manifests as embryonic death due to modifications to the sperm made by *Wolbachia* (J. H. Werren, 1997). CI occurs when a *Wolbachia*-free female mates with an infected male. All remaining crosses result in viable offspring. CI results in females with *Wolbachia* having greater reproductive success. This advantage, combined with *Wolbachia*’s vertical inheritance, causes the symbiont to spread through populations quickly (John H. Werren, Baldo, & Clark, 2008). *Wolbachia* has also been found to limit viral replication in various insects in a trait known as WMPB (Bian, Xu, Lu, Xie, & Xi, 2010; Dutra et al., 2016; Moreira et al., 2009). WMPB was first discovered in *Drosophila*, when insects infected with viruses exhibited longer lifespans if they also were infected with *Wolbachia* (Hedges, Brownlie, O’Neill, & Johnson, 2008). Eventually, it was determined that *Wolbachia* protects flies by reducing viral loads of the coinfecting virus (Teixeira, Ferreira, & Ashburner, 2008). This same result has been seen in *Ae. aegypti* for dengue, chikungunya, and Zika (Walker et al., 2011a) (Walker et al., 2011b), after artificially, but stably infecting them with *Wolbachia* (McMeniman et al., 2009; Walker et al., 2011a).
Currently, two main *Wolbachia* strains are being released in *Ae. aegypti* populations, wMel and wAlbB (A. A. Hoffmann et al., 2011; Nazni et al., 2019). wMel is derived from *Drosophila melanogaster* and has been demonstrated to be effective at limiting dengue virus infection in *Ae. aegypti* (Walker et al., 2011a). wAlbB is derived from *Aedes albopictus* and is similarly effective at blocking dengue virus in the vector (Joubert et al., 2016). Both strains have minimal effects on host fitness (Axford, Ross, Yeap, Callahan, & Hoffmann, 2016; Ary A. Hoffmann et al., 2014). wAlbB, however, is more tolerant to cyclical heat stress than wMel, suggesting that wAlbB may have higher success in warmer field environments (Ross et al., 2017).

The mechanism of *Wolbachia*-mediated pathogen blocking in mosquitoes is still not fully understood. From a range of studies, it is clear that the trait is likely multifaceted (Lindsey, Bhattacharya, Newton, & Hardy, 2018). Without the ability to genetically modify *Wolbachia* genes, much of the focus has been on identifying the effects of viral blocking in the mosquito. Processes in the mosquito such as immunity, nutrient competition, RNA translation and replication, and cellular stress are affected by *Wolbachia* and may assist with viral blocking (Ford et al., 2020; Geoghegan et al., 2017; Moreira et al., 2009; Rainey et al., 2016; Rancès, Ye, Woolfit, McGraw, & O’Neill, 2012; White et al., 2017). Multiple studies have shown that *Wolbachia* upregulates immune gene expression in *Ae. aegypti* (Moreira et al., 2009; Rancès et al., 2012). This priming of the immune system in *Ae. aegypti* may increase its basal immune protection, allowing the mosquito to have greater control over viruses it subsequently encounters during blood feeding. Another suggested basis of blocking is competition for cholesterol. Both dengue virus and *Wolbachia* depend on the vector’s cholesterol for survival and replication (Geoghegan et al., 2017; Heaton et al., 2010). Viral RNA translation and/or replication appears to be limited when *Wolbachia* is present (Rainey et al., 2016), possibly through alteration of the host’s ribosome that viruses use to replicate (White et al., 2017). This change may make the ribosome unsuitable for viruses (Lindsey et al., 2018). *Wolbachia* induces oxidative stress that
produces antimicrobial peptides in the host, inhibiting dengue virus proliferation (Pan et al., 2012). Last, *Wolbachia* increases expression of antioxidant proteins resulting in cellular stress that is counteracted by the production of reactive oxygen species (Brennan, Haukedal, Earle, Keddie, & Harris, 2012). Reactive oxygen species activate signaling pathways such as the extracellular signal-regulated kinase pathway (Thannickal & Fanburg, 2000), that has been demonstrated to increase viral protection in mosquito cells (Xu et al., 2013).

Regardless of the mechanism, blocking strength has been shown to correlate with relative *Wolbachia* densities (Amuzu & McGraw, 2016; Chouin-Carneiro et al., 2020; Chrostek et al., 2013; Iturbe-Ormaetxe, Walker, & O’Neill, 2011; Rainey et al., 2016). *Wolbachia* density could therefore be used to help study the basis of pathogen blocking, and indeed other *Wolbachia*-associated traits, if we could generate high and low-density lines in mosquitoes. Previous studies have compared closely related (Woolfit et al., 2013) *Wolbachia* strains, known to vary in their densities such as *w*Mel and *w*MelPop (Walker et al., 2011a), but such approaches are confounded by effects of virulence-associated with the latter strain. Similarly, comparing the same strain across different vectors where relative densities may vary, includes any confounding effects of other genetic differences between vectors, not specifically due to those that control relative *Wolbachia* density (Ikeda, Ishikawa, & Sasaki, 2003; McGraw, Merritt, Droller, & O’Neill, 2002). For example, *Ae. albopictus* is naturally co-infected with two *Wolbachia* strains, *w*AlbA, and *w*AlbB, that have a relative abundance of 1:10 in the native host (Dutton & Sinkins, 2004). When transinfected into *Ae. aegypti*, however, the relationship reverses, with *w*AlbA exhibiting a greater relative density than *w*AlbB (Ant, Herd, Geoghegan, Hoffmann, & Sinkins, 2018). Our goal in this study was to create genetically similar, independent lines of *Ae. aegypti* with stable and distinct differences in their *Wolbachia* densities from a single original strain of *Wolbachia*. By limiting *Wolbachia* strain and vector genetic diversity in the system, we could more easily identify the basis of density determination as well as study how density affects expressions of
WMPB and other Wolbachia-mediated traits. Here we utilized multiple approaches involving artificial selection, inbreeding, and tissue-based correlation to create predictably high and low-density lines or individuals.

3.3 Methods

3.3.1 Ae. aegypti rearing

Artificial selection was carried out on Ae. aegypti infected with the wMel strain of Wolbachia recently collected from the field in Cairns, Australia (Walker et al., 2011b). The tissue correlation and inbreeding experiments carried out several years hence with wAlbB infected Ae. aegypti obtained from Zhiyong Xi (Michigan State) (Xi, Khoo, & Dobson, 2005) as the wMel strain was no longer available due to MTA restrictions. For all experiments, mosquito eggs were hatched in 40 × 30 × 8 cm plastic trays with 3 liters of autoclaved reverse-osmosis water and fed Tetramin fish food (Melle, Germany) ad libitum. Larvae were maintained at a density of ~250 per tray. Populations of ~300 adult mosquitoes were housed in 18 x 18” square breeding cages (BioQuip). Dental wicks were used to feed a continuous supply of 10% sucrose. Mosquitoes were fed human blood (BioIVT) warmed to 37°C using an artificial feeder (Hemotek) at 9-11 days of age.

3.3.2 Experimental design

3.3.2.1 Artificial Selection Experiment

We first employed artificial selection regime in an attempt to create mosquito lines with increased whole-body relative densities of Wolbachia with the goal of studying the basis of DENV blocking. Experiments were carried out on lines within 3 generations of field collection to maximize vector genetic diversity. A total of 480 blood-fed wMel Ae. aegypti isofemales were placed in 70mL plastic cups (Sarstedt). Eggs were collected from the individuals using moist filter
paper. Females that laid eggs were then collected for DNA extraction and Wolbachia density measurement (below). Eggs were pooled from the three females exhibiting the highest densities to start three new populations (S1, S2, S3), each with ~300 individuals. In parallel, three control cages (C1, C2, C3), also with ~300 individuals each, were created by randomly selecting the offspring from females.

In each successive selection round, all lines were blood-fed 6-8 days post-eclosion by a human volunteer to instigate egg growth (ethics permit number CF11/0766-2011000387). 12-16 hours after blood-feeding, random samples of 80 females were taken from each line, housed individually in 70mL cups (Sarstedt), and provided a 10% sucrose solution. Wetted filter paper (Watman) provided a substrate for egg-laying. After three days, cups were checked for eggs. Dead females and those that laid <10 eggs were discarded. Eighty females (9-11 days of age) were isolated for eventual qPCR for each of the three cages and assessed for relative Wolbachia densities. Females were ranked by Wolbachia density, and eggs from the highest density females were pooled to seed the next generation for the individual line/cage. The number of females used to continue the next generation was constant for all three cages. A similar process was used for the three control cages, except females and their offspring were randomly chosen to seed the subsequent generation. The selection regime was carried out for a total of 4 generations.

3.3.2.2 Inbreeding Experiment

We also employed an inbreeding approach to see if we could generate lines with distinct and predictable relative Wolbachia densities (high vs. low). Before inbreeding, wAlbB females were outcrossed for three generations to Wolbachia-free male mosquitoes obtained from Monterrey, Mexico (Pablo Manrique-Saide, Universidad Autóonoma de Yucatán) to increase genetic diversity. To create inbred lines, we pooled the eggs collected from 41 single pair crosses,
ultimately leading to eight stable, independent lines. The original 41 P₁ females were dissected for ovaries and the remaining carcass at 15-17 days of age, or ~6 days post feed and egg-laying. We fed the eight inbred lines at each generation and then collected their eggs in 70 ml oviposition cups containing moist filter paper. Egg papers were hatched independently for each line, and adults were reared in cages (as above). After 8-9 generations of inbreeding, we carried out tissue dissection and relative Wolbachia density estimates for 24-25 females per line as in the parental generation. After Wolbachia quantification, we focused our subsequent rearing efforts on four lines, the two highest and two lowest lines with respect to relative Wolbachia densities in the carcass. Finally, at generations 11-13, we dissected ovary and carcass tissues again for comparison of relative Wolbachia densities to the P₁ and F₈₋₉ generations.

3.3.2.3 Tissue Correlation Experiment

After failing to select for high and low relative Wolbachia densities across generations by selection or inbreeding, we sought to determine whether we could predict Wolbachia density in the carcass or specific tissues based on first screening the mosquito legs. In brief, we dissected and pooled all legs from individual mosquitoes and then either kept the remainder of the body (minus gonads) or specific tissues including the midgut and the salivary glands for subsequent relative Wolbachia density determination as per below. All dissections were carried out at 15-17 days of age, or ~6 days post feed. Each experiment was based on 25-40 individuals.

3.3.3 Dissections and DNA extraction

In our artificial selection experiment, females were placed on a 96-well plate (VQR Lab Advantage) with 50 μl of extraction buffer (10 mM Tris buffer, 1 mM EDTA, 50 mM NaCl, and proteinase K) and a 2-mm glass bead. Plates were homogenized with a MiniBeadbeater-96 (Bio Spec) for 90 sec., centrifuged at 3220g for 3 mins and then incubated at 58°C for 30 sec. and at
96°C for 5 min. In our inbreeding and tissue correlation experiments, females were cold-anesthetized and dissected in 1x phosphate-buffered saline (PBS). Tissues were collected and placed in a 2 ml tube with 50 μl of PBS and a 3-mm glass bead. Dissected tissues were stored at -80°C until processing. To extract DNA, tubes were filled with 50 μl of extraction buffer. Samples were homogenized with a bead ruptor (OMNI International) for 90 sec., centrifuged at 2000g for 2 mins and then incubated at 56°C for 5 min and at 98°C for 5 min. A final centrifugation step was performed at 2000g for 2 mins to pellet any remaining mosquito tissue. Samples were diluted 1:10 using DNase/RNase free water prior to quantification.

3.3.4 Wolbachia quantification

Relative Wolbachia density was quantified through qPCR using Livak’s method (Livak & Schmittgen, 2001). In the artificial selection experiment, we used the primers TM513 and the mosquito ribosomal subunit protein S17 (RPS17). Primers: TM513_F (5’-CAATAAGCTCTTTGCTGCTG) and TM513_R (5’-GGGTGTTAAGCAGAGTTACGG), as well as mosquito primers RPS17_F (5’-TCCGTGGATCTCTCATCAAGCT) and RPS17_R (5’-CACTTCCGGCACGTAGTTTGC) (Ford et al., 2019). We also used a fluorescent probe for TM513 and RPS17. Probes: TM513 probe (5’-Lc640-TGAAATGGAAAAATTGGGCGAGGTAGTGG-Iowablack) and RPS17 probe (5’-FAM-CAGGAGGAGGAAACGTAGCGCAG-BHQ1) via qPCR on a LightCycler 480 (Roche), using the equation $2^{-\Delta \Delta C_{t}}$. The artificial selection experiment used a 10μL solution of 5μL of Lightcycler 480 Mastermix (Roche), 0.25 μL of each RPS17 primer, 0.1 μL of RPS17 probe, 0.3 μL of each TM513 primers, 0.3 μL of TM513 probe, 2.5μL of nuclease-free water and 1uL of template DNA.

For the inbreeding and tissue correlation experiments we also used the primer RPS17, but instead of TM513, we used previously published primers for wAlbB in an ankyrin repeat domain
gene (Axford et al., 2016); Primer: wAlbB_F (5’- CTTACCTCCTGCACAACAA) and wAlbB_R (5’-GGATTGTCCAGTGGCCTTA). All qPCR was carried out on a LightCycler 480 (Roche), using the equation $2^{-\frac{wAlbB}{RPS7}}$. Samples from inbreeding and tissue experiments were as follows; a total volume of 10μL per reaction, each containing: 5μL of 2x PerfeCTa SYBR Green SuperMix (Quantabio), 0.2μL of each forward and reverse primers (10μM), 2.6μL of nuclease-free water, and 8μL of template DNA. The qPCR temperature profile for both experiments included denaturation at 95°C for 5 min, 45 cycles of 95°C for 10 sec, 60°C for 15 sec and extension at 72°C for 10 sec, followed by a melt curve analysis.

3.3.5 Statistical analysis

Statistical analysis for the artificial selection study was performed in SPSS Statistics for Windows (IBM, Version 24.0). Density values were log10-transformed to reduce skewness. Statistical analysis for the inbreeding and tissue correlation experiment was performed in GraphPad Prism version 9.1.0 for Windows, GraphPad Software, San Diego, California USA. Data were checked for normality before performing analysis and transformed by log + 1 when necessary. All relative densities when depicted in scatter plots were plotted on a log axis. Fitted regression lines, although linear, can therefore appear curved.

3.4 Results

3.4.1 Artificial Selection Experiment

To determine whether artificial selection could be used to increase relative Wolbachia densities, Ae. aegypti mosquitoes were exposed to a selection regime for five generations. Wolbachia density was modeled using a mixed-effect model with generation and treatment as a fixed factor and line as a random factor nested within treatment. While treatment alone was not
significant, there was a significant effect of generation (F=17.39, df=4, p=0.001) and line (F=4.19, df=2, p=0.015), typically densities were highest at generation 1, reached a minimum at generations 3-4, and then began climbing during generation 5 (Figure 3.1). We also saw significant interactions between line and generation (F=4.86, df=8, p<0.0001) and treatment and generation (F=10.42, df=4, p<0.0001) (Figure 3.1). Both treatments had the parabolic trend described above, but our control lines decreased in *Wolbachia* densities faster than our artificially selected lines. The control lines reached a minimum at generation 3. Of note is the increase in *Wolbachia* density across the board from the base population in all treatments and control lines. Regardless of these interesting patterns, the selection regime did not increase density in the selected lines compared to controls. Repeating the statistical analysis with only generation 5 (endpoint) data also revealed no significant difference between selection and control-treated mosquitoes (F=0.325, df=1, p=0.60), but there was a significant difference between lines (F=28.40, df=4, p<0.0001). Control line 3 had the highest densities, while control line 1 had the lowest. In summary, we could not significantly shift *Wolbachia* densities based on whole-body estimates via artificial selection.

![Figure 3.1: Relative Wolbachia density for parental (G0), control (C1-3) and artificially selected (S1-3) lines at each generation (G1-5). Density is expressed as the number of Wolbachia TM513 gene copies normalized to the number of rps17 gene copies. Error bars represent +/-1 SD.](image-url)
In the original P₁ generation, we measured relative Wolbachia densities in ovaries and carcasses of isofemales 6 days post blood feeding and post egg collection (15-17 days of adulthood). Relative densities ranged from ~25 to ~195 and from ~0 to ~41 in the ovaries and carcass, respectively (Figure 3.2). This equated to a mean 2.3-fold higher density in ovaries than in carcass (P<0.0001) (Figure 3.2). We reassessed ovary and carcass densities after an additional 8-9 generations of rearing in our 8 remaining maternal lineages that survived the inbreeding process. Summing across lines, we saw no change in ovary densities (P=0.073) (Figure 3.3A) and a significant decrease in the carcass densities (P<0.0001) (Figure 3.3B) density compared to P₁.

When examining isofemale lines individually, we found that ovary density in isofemale lines #1 (P=0.0013) and #2 (P=0.0057) decreased, and line #8 (P<0.0001) increased compared to P₁. When comparing individual maternal lines to each other at F₈₉, we found that line #8 was higher than lines #1-6 (P<0.0001), and line #7 was higher than lines #1-3 (P<0.0001) and #4 (P=0.013) (Fig. 3.4A). Also, line #6 was higher than lines #1 (P=0.0016) and #2 (P=0.0060), and line #5 was higher than lines #1 (P=0.0024) and #2 (P=0.0085) (Figure 3.4A). For carcass densities, we found a decrease between all lines except #7 compared to P₁ (P<0.0001) (Figure 3.4B). Between F₈₉ lines, we found that line #7 was higher than lines #1-6 (P<0.0001) and #8 (P=0.0059) (Figure 3.4B). Also, for the carcass densities, line #8 was higher than lines #4 (P=0.0001), #6 (P=0.034), and #2 (P=0.012) (Figure 3.4B). At generations F₁₁-₁₃, we reassessed lines with the lowest (#’s 4 & 6) two and highest (#’s 7 & 8) two carcass density values. We found a significant increase in carcass density for lines 4 (P<0.0001) (Figure 3.5A) and 6 (P<0.0001) (Figure 3.5B). Line 8 increased in carcass density (P=0.0039) (Figure 3.5C) while line 7 decreased (P=0.008) (Figure 3.5D). All ovary densities, lines 4 (P=0.0035) (Figure 3.6A), 6 (P<0.0001) (Figure 3.6B), 8 (P=0.0005) (Figure 3.6C), 7 (P=0.0002) (Figure 3.6D) exhibited a decrease. In summary, we saw a decrease in relative
carcass densities after 8-9 generations of inbreeding and created lines with distinct *Wolbachia* densities. However, we could not maintain lines at high and low densities over multiple generations suggesting that inbreeding is not an avenue to create stable and distinct densities through time.

Figure 3.2: Relative *Wolbachia* densities (*ankyrin repeat domain to rps17*) in the ovaries and the carcass of *Ae. aegypti* in the parental generation (*P*₁), *n*=39, *P* <0.0001. Bars indicate tissue means ± SE; **** *P* ≤ 0.0001.
Figure 3.3: Relative Wolbachia densities (ankyrin repeat domain to rps17) in the parental generation (P₁) versus generations 8-9 (F₈₋₉). A. Wolbachia densities in the ovaries of P₁ versus the ovaries of F₈₋₉ in Ae. aegypti. B. Wolbachia densities in the carcass of P₁ versus the carcass of F₈₋₉ in Ae. aegypti. For A and B, P₁ n=39 and F₈₋₉ n=170. Bars indicate tissue means ± SE; ns, not significant; ****P ≤ 0.0001
Figure 3.4: Relative *Wolbachia* densities (ankyrin repeat domain to *rps17*) in each family line at generations 8-9 (families 1-8) versus the parental generation (*P*₁). **A.** *Wolbachia* densities in the ovaries. **B.** *Wolbachia* densities in the carcass. Different letters indicate significant differences between families based on Tukey’s test at *P* ≤ 0.05. *P*₁ has *n*=41 individuals and families 1-8 have *n*=24-25 individuals.
Figure 3.5: Relative *Wolbachia* densities (ankyrin repeat domain to rps17) in the carcass for the lowest two (4, 6) and highest two (8, 7) family lines at generations 8-9 (F$_{8,9}$) compared to their densities at generations 11-13 (F$_{11,13}$). **A.** *Wolbachia* densities for the line with lowest carcass density at F$_{8,9}$ versus F$_{11,13}$. **B.** *Wolbachia* densities for the line with second-lowest carcass density at F$_{8,9}$ versus F$_{11,13}$. **C.** *Wolbachia* densities for the line with highest carcass density at F$_{8,9}$ versus F$_{11,13}$. **D.** *Wolbachia* densities for the line with second-highest carcass density at F$_{8,9}$ versus F$_{11,13}$. For A and B n=~25 individuals. In Figure C, 8 F$_{8,9}$ n=25 and at F$_{11,13}$ n=12. In Figure D, n=24 individuals. Bars indicate tissue means ± SE; **P ≤ 0.001; ****P ≤ 0.0001
Figure 3.6: Relative *Wolbachia* densities (ankyrin repeat domain to rps17) in the ovaries for family lines densities at generations 8-9 (F8-9) compared to their densities at generations 11-13 (F11-13). A. *Wolbachia* densities for line 4 at F8-9 versus F11-13. B. *Wolbachia* densities for line 6 at F8-9 versus F11-13. C. *Wolbachia* densities for line 8 at F8-9 versus F11-13. D. *Wolbachia* densities for line 7 at F8-9 versus F11-13. In Figure A n=25. In Figure B, 6 F8-9 n=24 and at F11-13 n=25. In Figure C, 8 F8-9 n=25 and at F11-13 n=12. In Figure D, n=24 individuals. Bars indicate tissue means ± SE; **P ≤ 0.001; 0.001<***P < 0.0001; ****P ≤ 0.0001
3.4.3 Within Individual Tissue Correlation

We measured relative *Wolbachia* densities in legs, carcass (body minus ovaries), salivary glands, and midgut ~6 days post blood feeding (15-17 days of adulthood) to see whether leg densities could be used to accurately predict relative *Wolbachia* densities in the remaining carcass and specific tissues in single individuals. Leg densities were significantly (P<0.0001) lower (1.6-fold) than that of the total body densities (Figure 3.7). Importantly, we found a positive correlation between leg and carcass densities (P=0.014) (Figure 3.8) with an R² of 0.24, indicating some ability to use legs to predict tissue densities. We also found a correlation between salivary gland and leg densities (P=0.043) and between midgut and leg densities (P=0.026), but our R² values, 0.084 (Figure 3.9A), and 0.10 (Figure 3.9B), respectively, showing poor predictive ability. We found no correlation between the salivary gland and midgut densities (P=0.64) (Figure 3.9C). For our leg and total carcass density dataset, we then binned the leg densities into categories of high or low based on the mid-point value of the total range in densities (16.09) and examined our accuracy in predicting high and low loads in the carcass, similarly binned based on the midpoint of their total density range (20.24). We found that we were accurate 70% of the time, correctly predicting relative load category in the carcass. Then if we, selectively focused on legs at the extreme ends of the density range (top and bottom quartile), we could improve our accuracy of prediction up to 91% of the time. Taken together, our results suggest that leg density estimates can be used to accurately predict carcass densities in *Wolbachia*, an approach that, while destructive, may be useful for studying the impact of *Wolbachia* on gene expression, viral loads, and metabolomics in the carcass when DNA is not being sampled from those tissues in parallel.
Figure 3.7: Relative Wolbachia densities (ankyrin repeat domain to rps17) in the legs and the carcass of Ae. aegypti. n=24, P<0.0001. Bars indicate tissue means ± SE; 0.001<***P < 0.0001.

Figure 3.8: Relationship between relative Wolbachia densities (ankyrin repeat domain to rps17) in the legs and the carcass of Ae. aegypti. n=24.

$p=0.014$

$R^2=0.24$
3.5 Discussion

We show that artificial selection and inbreeding are not effective strategies for isolating and creating genetically similar Wolbachia strain:mosquito infections, that exhibit stable differences in their symbiont densities. Having this ability to study the impact of Wolbachia density on symbiont-induced traits of interest is much needed in the field, particularly in a system where genetic modification of the symbiont is not yet available. As a partial solution, we have found that within generation predictions, from legs to the remainder of the mosquito body, may allow sufficient predictability to bin mosquitoes a priori into the categories of low and high...
densities. Such an approach offers means to carry out various -omics studies on the mosquito body where the appropriate processing could not involve the collection of DNA for density assessment.

There are several possible explanations for why both artificial selection and inbreeding were unable to shift *Wolbachia* densities. With respect to our artificial selection experiment, a decoupling of the whole-body density from that in the ovaries may explain our result. A previous study in the mosquito *Culex quinquefasciatus* has shown just such a disconnect (Emerson & Glaser, 2017). Second, with respect to both approaches, we may have lacked substantial genetic variation in either the *Wolbachia* or the host. Many studies have demonstrated that native hosts for *Wolbachia* have lower densities than artificially infected hosts (Bian, Zhou, Lu, & Xi, 2013; Miller, Ehrman, & Schneider, 2010; Osborne, Iturbe-Ormaetxe, Brownlie, O’Neill, & Johnson, 2012). Therefore, density is in part dictated by yet unknown genetic factors in the host organism that may include immunity (Rancès et al., 2012; Ye, Woolfit, Rancès, O’Neill, & McGraw, 2013) or other aspects of mosquito physiologies. In *Wolbachia*, there is a positive correlation between gene copy numbers in the Octomom region of the genome in *D. melanogaster*-derived strains, and density demonstrating that genetic factors in the bacterium also dictate loads (Chrostek et al., 2013; Chrostek & Teixeira, 2015). Additionally, wAlbB relative density was found to be similar across the singly infected *Ae. aegypti* line and when found in co-infection in the same vector with the wMel strain (Joubert et al., 2016), supporting our claim of *Wolbachia*’s genotype-based influences. Our selection and inbreeding experiments were carried out with different *Wolbachia* strain x mosquito population combinations; however, both were optimized for high genetic variation in the starting populations of the vector. *Wolbachia*, however, in both scenarios will have much reduced genetic diversity, having initially been created through a single or handful of females that became infected via artificial transinfection (Walker et al., 2011a; Xi et al., 2005).
Additionally, we know from laboratory culturing and resequencing experiments that *Wolbachia* tends to evolve very slowly (Woolfit et al., 2013), likely due in part to the constraints of extreme bottlenecks at each generation in the insect. In wasps (L. Mouton, Henri, Bouletreau, & Vavre, 2003) and flies (Correa & Ballard, 2012), inbred laboratory lines with *Wolbachia* have been shown to reduce variation in relative *Wolbachia* density. A recent study in *D. melanogaster* infected with *wMel*, showed that inbreeding caused *Wolbachia* densities in the whole body to reach a maximum in the host every 1-2 generations followed by an extremely low load in the next generation (Liu & Li, 2021). One study, in an *Ae. albopictus* line, recently colonized from the field, showed that *Wolbachia* densities have poor predictability across generations (Ahantarig, Trinachartvanit, & Kittayapong, 2008).

In both the artificial selection and inbreeding experiments, we saw a similar pattern of higher densities in individual lines returning to lower levels in a generation or two as with the Drosophila study above (Liu & Li, 2021). While we aimed to keep all environmental conditions (temperature, larval densities, etc) consistent across generations and experiments they could have also been a factor. Temperature can affect *Wolbachia* densities (Madhav et al., 2020; L. Mouton et al., 2003; Laurence Mouton, Henri, Charif, Boulétreau, & Vavre, 2007; Serbus et al., 2015), particularly in *wMel* (Ulrich, Beier, Devine, & Hugo, 2016) as compared to *wAlbB* (Ross et al., 2017). Similarly, larval crowding and *ad libitum* food delivery can limit *Wolbachia* densities (Dutton & Sinkins, 2004; Wiwatanaratananabutr & Kittayapong, 2009). High *Wolbachia* densities may cause detrimental effects on the host. A study in *Ae. aegypti*, specifically, has shown that high *Wolbachia* densities result in higher fitness costs (Ant et al., 2018). This is not surprising, as an intracellular symbiont with a much-reduced genome (Wu et al., 2004), that is highly dependent on its host for range of resources including cholesterol (Geoghegan et al., 2017) and nucleotides (Wu et al., 2004). For the host cholesterol is needed for cellular signaling (Kabouridis, Janzen, Magee, & Ley, 2000) and membrane stability (Eaton, 2008), while nucleotides are essential for
cellular energy and DNA synthesis (Wegener, 1996). When the virulent, overgrowing strain of wMelPop was transinfected from \textit{D. melanogaster} to \textit{D. simulans}, \textit{Wolbachia} loads were initially very high (McGraw et al., 2002). As they declined, so did the associated fitness effects in the novel host. Higher densities may also trigger a greater immune response (Kambris, Cook, Phuc, & Sinkins, 2009) that in turn regulates the \textit{Wolbachia} numbers (Deehan, Lin, Blum, Emili, & Frydman, 2021; Ye et al., 2013). Immune defense activities are themselves costly (Ahmed, Baggott, Maingon, & Hurd, 2002; Schwartz & Koella, 2004), which may explain a balancing act for hosts and a cycling of \textit{Wolbachia} loads, keeping \textit{Wolbachia} levels in check within a reasonable range, while not over-reacting to them.

Our results do show a correlation between the relative density of \textit{Wolbachia} wAlbB in the legs and the rest of the mosquito body (minus ovaries) that may be used with some accuracy to separate the remaining body into high and low relative density groups. Unfortunately, these correlations did hold for specific tissues like midgut and salivary glands that may be of special interest for questions about vector competence and vector susceptibility. Transcriptional studies have shown that immune gene expression varies across the midgut, salivary gland, and carcass (Bonizzoni et al., 2012; Sim, Ramirez, & Dimopoulos, 2012). There are also cells in the midgut epithelia and salivary glands that have higher densities of \textit{Wolbachia} than other tissues in the vicinity (Joubert et al., 2016) that may explain variation.

### 3.6 Acknowledgments

The authors would like to thank Zhiyong Xi and Pablo Manrique-Saide for providing the wAlbB and Wildtype \textit{Ae. aegypti} lines, respectively. We thank the McGraw lab for their support and helpful discussions with the methods and results of this study. We thank Fhallon Ware-Gilmore for her help in beautifying our figures. We also thank Heverton Dutra for his help during dissections.
3.7 References


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Chapter 4

Discussion and Future Directions

The mosquito *Aedes aegypti* is one of the deadliest animals in the world and its geographic range is expanding (Chandrasegaran, Lahondère, Escobar, & Vinauger, 2020; Kraemer et al., 2019). Unfortunately, we have no effective drugs or vaccines to treat or prevent the viruses the vector transmits (Merle et al., 2018). Instead, we depend on the biocontrol of *Ae. aegypti* to limit viral transmission. One essential tool in this vector control is the insect endosymbiont, *Wolbachia*. After *Wolbachia* was transinfected into *Ae. aegypti*, it was shown to cause Cytoplasmic Incompatibility (CI), and pathogen blocking in the vector. These two phenotypes are essential for biocontrol strategies as they can be used to crash *Ae. aegypti* populations by CI (‘population suppression’) or replace native mosquitoes with a vector that can limit viral replication (‘population replacement’). There is a positive relationship between *Wolbachia* densities and the strength of both CI and pathogen blocking (Amuzu & McGraw, 2016; Shropshire, Hamant, & Cooper, 2021).

Chapter 2 focused on comparing *Wolbachia* densities between tissues within and across generations of *Ae. aegypti*. The lack of predictability of tissue densities is possibly due to *Wolbachia*'s preferences for specific cellular environments. The bacterium's cellular preferences are why *Wolbachia* density studies see varying tissue densities in different mosquito species (Ant & Sinkins, 2018; Emerson & Glaser, 2017). There was a positive correlation between egg and ovary densities within a generation. During the embryonic stages, *Wolbachia* is found at low densities, and it is not until late development stages that densities quickly rise (Min & Benzer, 1997). There are no studies on why densities are found at low levels during early development. This trend could be occurring since cellular resources limit *Wolbachia* during the host's embryonic life stages but not as an adult. Further work needs to be conducted to understand
Wolbachia's transition from embryo to adult since we see a negative relationship between eggs and ovaries across a generation. One would expect a positive relationship between these tissues similar to the within generation data. Natural selection should select for Wolbachia genotypes that grow to high densities in the egg to ensure their transmission to the next generation. However, something is limiting Wolbachia's densities in the egg. Currently, the theory is that Wolbachia's dispersal in the egg's cytoplasm represents a fate map as to which tissues acquire specific amounts of the bacterium (Pietri, DeBruhl, & Sullivan, 2016). Further research should be conducted to learn about the connection between Wolbachia densities in the embryo to the adult.

In chapter 3, we attempted to create Ae. aegypti lines with stable high and low Wolbachia densities. However, we failed to maintain stable densities with artificial selection and inbreeding. We did see a similar cyclical trend in Wolbachia densities between both experiments. The cycle involved Wolbachia densities reaching a max threshold in the vector, followed by a decrease in quantity. What makes our result more interesting is that this same cycle has recently been found in Drosophila melanogaster (Liu & Li, 2021). Our result hints at the bigger problem, what is driving Wolbachia densities? Is it the host, or is it Wolbachia? One argument is that the host's immunity is limiting Wolbachia densities because at high amounts, Ae. aegypti sees the bacteria as pathogenic. In an over replicating Wolbachia strain, immunity increases with higher densities (Kambris, Cook, Phuc, & Sinkins, 2009). Additionally, in chapter 2, we saw a negative correlation between egg and ovary densities that could be occurring because the host is controlling densities. However, I believe that Wolbachia is mostly in control of densities. Wolbachia may be limiting densities unintentionally as higher quantities increase host fitness costs (Ant, Herd, Geoghegan, Hoffmann, & Sinkins, 2018). I believe that Ae. aegypti with high densities produce fewer eggs and are worse blood-feeders than mosquitoes with low densities. My inbred lines that were less likely to blood feed, lay eggs, and have their eggs hatch were also the ones with the highest densities. I was therefore likely selecting for lines with lower eggs at
each generation. One way to ensure this is the case is to repeat the inbreeding experiment and measure fecundity. Similarly, what might have decreased densities during the artificial selection experiment was high densities killing females before they could lay eggs.

Taken together, what does this mean for field releases of *Wolbachia*-infected *Ae. aegypti*. My data suggest that we cannot create *Ae. aegypti* with stable high *Wolbachia* densities through tissue correlation, inbreeding, and artificial selection. One exception and possible solution is using the legs and carcass to create a line with high densities. However, my data for legs and carcass only measured densities within a generation and not across, thus warrants further investigation. Regardless, I suggest that instead of creating lines with high *Wolbachia*, we should focus on finding a bacterial strain that naturally has higher densities. Current field releases have shown that densities have remained stable even after three years of release (Tantowijoyo et al., 2020). We also need to assess that this strain does not incur high fitness costs and has a reasonable fecundity rate if we plan to replace the native mosquito population. I would also examine how temperature will affect density levels. Due to densities being affected by many factors, we kept variables constant throughout the experiments. However, if we plan to release *Wolbachia*-infected mosquitoes, the temperature should not remain constant. Instead, I would repeat the inbreeding experiment with multiple *Wolbachia* strains and change rearing temperatures for the larvae and adults. As global temperatures are rising, we want to be sure that *Wolbachia* densities will not drop in the future.

*Wolbachia's* viral blocking can be attributed to many factors, but we are unsure what the critical mechanism is. In addition to our focus on creating lines with high densities for field releases. Our other priority should be to create low-density lines to learn more about arboviruses and *Wolbachia* since the bacterium is genetically intractable. By creating lines with varying *Wolbachia* densities, shown to be possible using the legs and carcass, we can test mosquitoes for viral blocking differences. For example, we know that *Wolbachia* and dengue virus uses similar
lipids (Koh et al., 2020). Will lines with high *Wolbachia* outcompete dengue for lipids? Are high and low lines using similar lipids? Answering these questions can give us insight into arboviral dynamics in *Ae. aegypti* and what are the essential nutrients for *Wolbachia* replication.

Through my work, I demonstrate that *Wolbachia* densities are more likely determined by stochastic factors, interactions with the environment, and local tissue-based effects (ie. nutrients, space, immunity, etc) than either the maternal mosquito genotype or shared environments in the embryo. Future studies should attempt to explore the physiology of *Wolbachia*:mosquito interactions at the level of the cell or tissue to dissect these alternate explanations. My research did find a method for accurately predicting *Wolbachia* densities in mosquito bodies from the leg tissue. This approach, while unable to generate stable predictable lines, could be used to bin carcasses into High and Low treatments for a range of non-DNA-based -omics approaches for the study of *Wolbachia*-mediated traits. While many questions remained unanswered, my research provides insight into how *Wolbachia* densities are being controlled and what this means for field releases.
References


Appendix

Appendix: Supplementary materials from Chapter 2

Appendix: Supplementary figure 1. Crossing point values of rps17 in the ovaries and the carcass of Ae. aegypti in the same generation of mothers (P1).

Crossing point values of rps17 in the ovaries and the carcass of Ae. aegypti in the same generation of mothers (P1). These data pertain to Figure 2.2. n=206, P<0.0001 (paired t-test). Bars indicate tissue means ± SE; ****P ≤ 0.0001.
**Appendix:** Supplementary figure 2. Crossing point values of *rps17* in the ovaries, carcass, and eggs of *Ae. aegypti.*

Crossing point values of *rps17* in the ovaries, carcass, and eggs of *Ae. aegypti.* These data pertain to Figure 2.4. One-way ANOVA *P*<0.0001; post-hoc Tukey’s test: ovaries versus eggs: *P*<0.0001; carcass versus eggs: *P*<0.0001; ovaries versus carcass: *P*<0.0001. n=18 individuals. Bars indicate tissue means ± SE; **** *P* ≤ 0.0001.
Appendix: Supplementary figure 3. Relative Wolbachia densities (ankyrin repeat domain to rps17) in the ovaries, carcass, and eggs of Ae. aegypti in replicate group 2.

Relative Wolbachia densities (ankyrin repeat domain to rps17) in the ovaries, carcass, and eggs of Ae. aegypti in replicate group 2. One-way ANOVA $P<0.0001$; post-hoc Tukey’s test: ovaries versus eggs: $P<0.0001$; carcass versus eggs: $P<0.0001$; ovaries versus carcass: $P=0.33$. n=18 individuals. Bars indicate tissue means ± SE; ns, not significant; ***$P \leq 0.001$; ****$P \leq 0.0001$. 
Appendix: Supplementary figure 4. Relative *Wolbachia* densities (*ankyrin repeat domain* to *rps17*) in the ovaries, carcass, and eggs of *Ae. aegypti* in replicate group 2.

Relative *Wolbachia* densities (*ankyrin repeat domain* to *rps17*) in the ovaries, carcass, and eggs of *Ae. aegypti* in replicate group 2. A. *Wolbachia* densities in the eggs versus the ovaries of *Ae. aegypti* in replicate group 2. B. *Wolbachia* densities in the eggs versus the carcass of *Ae. aegypti* in replicate group 2. n=18 individuals
Appendix: Supplementary figure 5. Crossing point values of *rps17* in the tissues of mothers (P₁) and daughters (F₁).

Crossing point values of *rps17* in the tissues of mothers (P₁) and daughters (F₁). These data pertain to Figure 2.6. A. Crossing point values of *rps17* of P₁ in *Ae. aegypti*. B. Crossing point values of *rps17* of F₁ in *Ae. aegypti*. Figure A has n=31 individuals while B has n=78 individuals. Bars indicate tissue means ± SE; ****P ≤ 0.0001
Appendix: Supplementary figure 6. Crossing point values of *rps17* in the eggs of mothers (P₁) and tissues of daughters (F₁).

Crossing point values of *rps17* in the eggs of mothers (P₁) and tissues of daughters (F₁). These data pertain to Figure 2.7. For P₁ eggs n=20 individuals, while for ovaries and carcass n=94 individuals. Bars indicate tissue means ± SE; ns, not significant; ****P ≤ 0.0001.
Appendix: Supplementary figure 7. Relative Wolbachia densities (ankyrin repeat domain to rps17) in the ovaries and the carcass of *Ae. aegypti* in the same generation of daughters (F1).

Relative *Wolbachia* densities (ankyrin repeat domain to rps17) in the ovaries and the carcass of *Ae. aegypti* in the same generation of daughters (F1). n=79, P=0.21 (paired t-test). Bars indicate tissue means ± SE; ns, not significant.
Appendix: Supplementary figure 8. Relationship between relative *Wolbachia* densities in the ovaries and the carcass of *Ae. aegypti* in the same generation of daughters (F$_1$). 

![Relationship between relative Wolbachia densities in the ovaries and the carcass of Ae. aegypti in the same generation of daughters (F$_1$). n=79.](image)

Relationship between relative *Wolbachia* densities in the ovaries and the carcass of *Ae. aegypti* in the same generation of daughters (F$_1$). n=79.