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Characterizing the Complex Relationship Between the Brown Widow Spider and Its Bacterial Endosymbiont, Wolbachia

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CHARACTERIZING THE COMPLEX RELATIONSHIP BETWEEN THE BROWN WIDOW SPIDER AND ITS BACTERIAL ENDOSYMBIONT, WOLBACHIA

by

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(Under the Direction of John Scott Harrison)

ABSTRACT

The brown widow spider, *Latrodectus geometricus* (C. L. Koch 1841), has been found to harbor the maternally inherited bacterial endosymbiont *Wolbachia pipientis* (Hertig and Wolbach 1924), but endosymbiont infection frequency varies widely among Southeastern US populations. *Wolbachia* is known to manipulate the reproduction of its hosts through male feminization, parthenogenesis, male killing, and cytoplasmic incompatibility. In brown widows, *Wolbachia* does not alter sex ratios, but any other effects the symbiont has on the spider are unknown. In my first chapter, I assess if there is linkage between *Wolbachia* infection and maternally inherited mitochondrial DNA (mtDNA) in three brown widow populations. I found no evidence of linkage between *Wolbachia* infection and mtDNA haplotypes, despite both being maternally inherited. This result is consistent with weak fitness manipulation by the endosymbiont on the host, and could explain the variable, and often low, population infection frequencies in brown widow populations. Lack of linkage could also be the result of common leakage events, in which the bacteria is randomly lost from one generation to the next. In my second chapter, I determine if *Wolbachia* can induce cytoplasmic incompatibility (CI) in the brown widow. I provide evidence that *Wolbachia* infection causes partial CI in the brown widow. Weak host effects, such as partial CI, is consistent with the lack of linkage between *Wolbachia* and mtDNA described in Chapter 1, as well as the variable infection frequencies among populations. In my last chapter, I

explore *Wolbachia* concentrations in brown widow body regions. I found that endosymbiont load did not differ among three body regions, indicating that any host effects are not tissue specific. *Wolbachia* load, however, does vary among maternal lineages. The observed variation in *Wolbachia* load among maternal lines should be tested as a possible cause of variation in CI levels among mating pairs. This study may help us better understand the relationship between evolutionary genetics and the strength of host manipulation by endosymbionts.

INDEX WORDS: Brown widow spider, Host-parasite interaction, *Wolbachia pipientis*, *Latrodectus geometricus*, Mitochondrial DNA, Life history, Population dynamics, Reproduction manipulation, Cytoplasmic incompatibility, Bacterial load

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by

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DEDICATION

I would like to dedicate this to my parents, Robbie and Robin, as well as my husband, Bryan. I love you all.

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I would like to think Scott Harrison, my advisor, for allowing me to explore the world of population genetics with his assistance and support. I would also like to thank my committee member, Edward Mondor, for his continuing support, words of encouragement, statistical insight, and overall life advice that has kept me afloat. Thank you to my committee member Joshua Gibson for generous giving his time to join my team in a time of need. Special thanks to Michelle Tremblay for helping in spider rearing and for becoming a friend. Maggie Howard, you are my rock. Finally, thank you to my Clemson family for constant encouragement and pushing me to continue.

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INTRODUCTION

When studying the history of life, it is important to look at symbiosis, or the living together of unlike organisms (De Bary 1879, Douglas 2010). Intracellular endosymbionts reside within the cells of their hosts (Zug and Hammerstein 2015). In invertebrates, especially arthropods, bacterial endosymbionts are abundant across taxonomic groups (Zchori-Fein and Bourtzis 2011). Endosymbionts can have a variety of significant effects on host fitness through mutualistic and parasitic relationships (Zug and Hammerstein 2015). *Wolbachia pipientis* (Hertig and Wolbach 1924) is a well know bacterial endosymbiont that has been found to have a wide range of effects on its host.

Wolbachia is a gram-negative intracellular alphaproteobacterium that infects nematodes and arthropods (Werren *et al*. 2008). Variants of this endosymbiont have been divided into eight supergroups (A-H), with super groups C and D being commonly found in filarial nematodes and the other six supergroups being found predominantly in arthropods. A study of 63 arthropod species showed that 76% harbored *Wolbachia* (Jeyaprakash and Hoy 2000). Both within and among supergroups, *Wolbachia* is known to have variants that induce different host effect, with related forms potentially producing different host compatibility types (O'Neill *et al*. 1992, Rousset *et al*. 1992). Supergroups A and B are the most common in arthropods (Werren *et al*., 2008, Baldo *et al.* 2007). Supergroup F is a unique supergroup that has been found in termites (Lo and Evans 2007), filarial nematodes (Casiraghi *et al*. 2005), bed bugs (Hosokawa 2010), bush crickets (Panaram and Marshall 2007), lice (Covacin and Barker 2007), and Southern African scorpions (Baldo *et al*. 2007). The effects of supergroup F on their hosts unknown, except for Hosokawa (2010) finding that the *Wolbachia* aided in nutrient acquisition of vitamin B that promoted successful egg development in bed bugs.

Wolbachia has been found to have a wide range of host manipulations. This includes reproductive manipulations, such as turning haploid parasitic wasp eggs, which turn into males, into diploid eggs, which become females with no fertilization needed (Russell and Stouthamer 2011). Nutritional acquisition has also been seen in bedbugs, in which *Wolbachia* supplies vitamins needed for development (Hosokawa 2010). Viral protection, such as in drosophila where infected flies are more resistant to RNA viruses has also been seen (Hedges *et al.* 2008).

Wolbachia is maternally inherited through the cytoplasm of the egg in the same way that mitochondria are inherited (Shoemaker *et al*. 2000). Several types of reproductive manipulations have evolved to increase the number of infected females in the host population (Werren 2008). Reproductive manipulations include: male feminization, parthenogenesis induction, male killing, and cytoplasmic incompatibility (CI) (Werren 2008). Male feminization is where genetic males develop as functional females (Werren 1997, 2008). Parthenogenesis results in the development of unfertilized eggs laid by virgin haplodiploid females (Russell and Stouthamer 2011, Werren 2008). Male killing is where male embryos laid by an infected mother do not develop (Sakamoto 2011, Werren 2008). Cytoplasmic incompatibility results in a lack of embryonic development when infected males mate with uninfected females or females infected by a different strain (Turelli 1994, Hoffman and Turelli 1997). Infected females can mate with either infected or uninfected males (Turelli 1994, Hoffman and Turelli 1997). Infected females have two mating options for viable offspring, while uninfected females can only mate with uninfected male to produce viable offspring. If CI is strong, the increased relative fitness of infected females will drive *Wolbachia* to spread rapidly and reach fixation within the population. With these potential manipulations, understanding the biology of the spider is limited until the relationship of the endosymbiont and spider is better understood or resolved.

Latrodectus geometricus (C. L. Koch 1841), or the brown widow spider, is a species of widow spider that is thought to be native to South Africa, but can now be found on every continent except Antarctica (Garb *et al.* 2004, Brown *et al.* 2008). *Wolbachia pipientis* was recently identified in the brown widow (Arrington 2014). Brown widows were first documented in South Florida in the 1930s and its distribution was limited to South Florida for around 50 years, but the range expanded to north Florida in the 1980's and further across the southeast in the late 1990's and early 2000's (Brown *et al*. 2008). The spiders can now be found in Georgia, Alabama, South Carolina, Mississippi, Louisiana, and Texas (Brown *et al*. 2008). It has also appeared in California (Garb *et al*. 2004) and Hawaii (Pinter 1980).

Arrington (2014) sampled multiple areas in the Southeastern US and determined *Wolbachia* infection status in different introduced brown widow populations. It was concluded that infection is not fixed in any one population, as infection frequency ranged from 20% in Bulloch County, Georgia to 90% in Miami, FL (Arrington 2014). The range and distribution pattern of *Wolbachia* in Southeastern US brown widow populations provides an ideal situation to test influences on host interactions. This infection could be recent and we are just seeing the beginning of *Wolbachi*a frequency increase. This infection could also be an old infection and is becoming lost over time. There could also be different selective pressures in novel areas that cause the bacteria to be selected against.

The goal of my thesis was to bring insight into the host-endosymbiont relationship between brown widows and *Wolbachia*. To address this, I had three different objectives. First, I wanted to determine if there is a linkage between *Wolbachia* and the mitochondrial DNA of the spiders. Second, I wanted to determine if *Wolbachia* induces cytoplasmic incompatibility in the

brown widow. Lastly, I wanted to determine if there is a difference in bacterial load between body regions.

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CHAPTER 1

Defining the Relationship of Mitochondrial DNA and *Wolbachia pipientis* in Three Populations of *Latrodectus geometricus*

ABSTRACT

The brown widow spider, *Latrodectus geometricus*, was first documented in the United States in South Florida in 1935 and has recently expanded its range in the Southeastern US. The brown widow has recently been found to harbor the bacteria *Wolbachia pipientis.* This maternally inherited bacterium has been known to cause a selective sweep of maternally inherited mitochondrial DNA. This study describes temporal and spatial haplotype and *Wolbachia* frequency variation within non-native brown widow populations in the Southeastern US. *Wolbachia* frequency increased in Louisiana by 19.2% over a 7-year period, while Georgia populations showed an 8.1% decrease in frequency over three years. There was no correlation between any mitochondrial haplotype and *Wolbachia* infection. Measures of haplotype diversity did not differ between infected and uninfected populations and did not change over time within populations. These results are consistent weak effects of *Wolbachia* on the host and that the host-symbiont association in this case is old and is being lost through the reduction of selective pressures.

INTRODUCTION

The brown widow spider, *Latrodectus geometricus*, is thought to have originated in South Africa, it can now be found on every continent except Antarctica (Brown *et al*. 2008). It has been rapidly expanding its range in the United States since the 1990's, after being found in southern

Florida in 1935 (Vetter *et al*. 2012). The spider's distribution has expanded up the Florida peninsula after introduction and can now be found in Georgia, Alabama, South Carolina, Mississippi, Louisiana, and Texas (Brown *et al*. 2008). The spider also appeared in California (Garb *et al*. 2004) and Hawaii (Pinter 1980).

Wolbachia is an *alphaproteobacterium* that infects a broad range of arthropods and filarial nematodes (Werren *et al.* 2008). It is maternally inherited (Zug and Hammerstein 2015) and spreads through reproductive manipulations that increase the reproductive fitness of infected females (Engelstädter and Hurst 2009). This is the result of one of four manipulations that have evolved: male feminization, parthenogenesis, male killing, and cytoplasmic incompatibility (Werren 2008). The most common of these reproductive manipulation strategies is cytoplasmic incompatibility (CI), which results in embryonic death between infected males and uninfected females, while the reciprocal cross is viable (Hoffmann and Turelli 1997). Parthenogenesis occurs when unfertilized eggs laid by a virgin haplodiploid female develop into functional progeny, however this is unlikely, as brown widows are not a haplodiploid species (Russell and Stouthamer 2011, Werren *et al.* 2008). Male feminization occurs when functional females develop from genetic males (Werren 1997, 2008). Male killing is when there is a loss of male embryos laid by an infected mother (Sakamoto 2011, Werren 2008). Male killing and feminization have not been observed in *L. geometricus* (Arrington 2014)

The brown widow spider has been found to harbor the bacterial endosymbiont *Wolbachia pipientis* (Arrington 2014). *Wolbachia* found in the brown widow is a member of supergroup F, which can also be found in filarial nematodes (Casiraghi *et al*. 2005), termites (Lo and Evans 2007), bush crickets (Panaram and Marshall 2007), lice (Covacin and Barker 2007), South African scorpions (Baldo *et al*. 2007), and bedbugs (Hosokawa 2010). The strain of *Wolbachia*

that infects the brown widow is unique within supergroup F (J.S. Harrison unpublished data). Arrington (2014) found that *Wolbachia* infection ranged from 20% to 90% in tested populations in the Southeastern United States, showing that the infection is not fixed and varies among locations (Figure 1.)

With heritable endosymbionts having the ability to manipulate the ecology and evolution of their hosts (McFall-Ngai *et al*. 2013), an understanding of brown widow life history and evolution is incomplete without incorporation of host-endosymbiont interactions. *Wolbachia* has been found to influence the genetic diversity of maternally inherited mitochondria, which could result in a difference in host fitness (Hurst and Jiggins 2005). As *Wolbachia* and mitochondria are both cytoplasmically inherited, there is an opportunity for linkage. That is, in populations where infected individuals gain a fitness advantage, the mitochondrial genomes (mtDNA) of the infected individuals will hitchhike with the spreading *Wolbachia*. This hitchhiking will in turn will reduce mtDNA haplotype diversity (Narita *et al*. 2006, Charlat *et al.* 2009, Atyame *et al.* 2011). With this replacing of haplotypes, populations that are infected with *Wolbachia* can display a different or fewer mitochondrial lineages than uninfected ones (Jiggins 2003, Hurst and Jiggins 2005). *Wolbachia* infected populations have been found to have lower amounts of mtDNA diversity than uninfected populations (Shoemaker *et al*. 2003). These changes can result in fitness differences between infected and uninfected individuals, which could be associated with fixation of slightly deleterious or beneficial mitochondria haplotypes (Ballard and Whitlock 2004). If gene flow was common between populations both *Wolbachia* frequency and mitochondria haplotype frequency would be similar in the absence of selective forces (Dyer and Jaenike 2004, 2005). *Wolbachia* infection frequency in brown widows ranged from 20% in Statesboro, GA to 90% in Miami-Dade, FL, suggesting little to no gene flow and that brown

widow populations have isolated distributions (Arrington 2014). This wide range of *Wolbachia* frequency gives a unique experimental system to test the relationship between *Wolbachia* infection and mtDNA variation at different frequencies of infection.

The aim of this study is to determine if there is linkage between *Wolbachia* infection and mtDNA in the brown widow. I ask the following questions: 1) Does mtDNA variation differ between *Wolbachia* infected individuals compared to uninfected individuals within and among non-native brown widow populations, 2) does mtDNA variation and *Wolbachia* infection frequency change over time during the establishment of non-native brown widow populations? I hypothesize that mtDNA variation will be lower among *Wolbachia* infected individuals than uninfected individuals within and among non-native brown widow populations. *Wolbachia* spreads via maternal cytoplasmic transmission, which allows mtDNA to hitchhike (Ballard and Whitlock 2004). This leads to increases in the frequency of a single mtDNA haplotype (Turelli and Hoffman 1995), which is considered a hitchhiking effect, or loss of variation, in the mtDNA (Xiao *et al*. 2011). I also hypothesize that mtDNA variation has decreased and *Wolbachia* infection frequency has increased over time during the establishment of non-native brown widow populations.

METHODS

Testing for Wolbachia

Brown widow spiders were collected from three populations on different years. Spiders from New Orleans, LA were collected during 2006 (n= 20), 2009 (n= 27), and 2013 (n= 31). Spiders were also collected from Miami, FL in 2013 (n=33) and Statesboro, GA during 2013 (n= 67) and 2016 (n= 69). To avoid sampling related individuals, spiders were collected from various structures at each location. Many of the samples collected prior to 2016 were previously

extracted and the DNA frozen (Arrington 2014). For the 2016 samples and prior to 2016 samples who did not have previously extracted DNA (specimens were labeled and stored in 95% ethanol), a whole leg was removed for DNA extraction (Arrington 2014). ZR Genomic DNA™-Tissue Microprep kit was used to perform the DNA extraction by following the manufacturers protocol (Zymo Research). Presence of *Wolbachia* was determined by Polymerase Chain Reaction (PCR) using the *Wolbachia* specific primer for the fructose-bisphosphate aldolase (FbpA) gene, which has been found to amplify in all known strains of *Wolbachia* (Vanthournout *et al*. 2001, Simões *et al*. 2011). The PCR protocol from Simões *et al.* (2011) for FbpA (F. GCTGCTCCRCTTGGYWTGAT) was used in PCR and the products were run on a 1% agarose gel. The presence or absence of PCR product for the FbpA gene determined *Wolbachia* infection status.

Wolbachia frequency

Wolbachia frequency was found for all three populations in each year. For the populations where temporal samples were taken and available, two statistical approaches were used to test for differences in *Wolbachia* infection frequency between years. A Z-test was conducted to determine if there was a difference in *Wolbachia* infections among different populations and years. A chi-square test of equal frequencies within time points for each population was also calculated using the statistical software JMP (SAS Institute Inc. 2009). Temporal variation was not assessed for the Florida samples, as samples were only collected for one year.

mtDNA sequencing

Mitochondrial DNA (mtDNA) was amplified in all the samples that were tested for *Wolbachia*. PCR was performed using a mtDNA specific primer that targets a 650 bp region of the cytochrome c oxidase I (COI) gene. The samples were tested for amplification by gel electrophoresis on a 1% agarose gel. The PCR products were then cleaned using Exonuclease I-Shrimp Alkaline Phosphatase (ExoSAP) protocol (New England BioLabs). The cleaned samples were then sent to the University of Georgia's Georgia Genomics Facility for sanger DNA sequencing using the forward COI primer.

Haplotype differentiation

The sequences were edited using Sequencher software and edited (Gene Codes Corporation). Haplotypes were determined based on a 100% minimum match percentage. Haplotype diversity, nucleotide diversity, and Θ_W were calculated for infected and uninfected groups for each sampling year using the program DNAsp v6 (Rozas *et al.* 2017). Haplotype diversity (also known as gene diversity, Hd) represents the probability that two randomly sampled alleles are different. Nucleotide diversity, or PI (π) is a measurement of the sequence diversity of the nucleotides (Nei and Li 1979). This measurement is based on single nucleotide polymorphisms (SNPs) and is the probability that nucleotides from different sequences of the same gene are different. The Watterson estimator was used to calculate theta based on *S* (Θw), or a measure of population mutation rate, by looking at the number of segregation sites per sequence (Watterson 1975).

Phylogenetic Analysis

Sequences were aligned using ClustalW (Larkin *et al.* 2014). A gene tree was estimated using Bayesian analysis in the program MrBayes (Huelsenbeck and Ronquist 2001). Parameters were set to HKY85+I+gamma. Branch confidence was assessed using Markov chain Monte Carlo with 100000 generations, sampling trees every 100 generations, with a 500 tree burn-in.

RESULTS

The Louisiana population had a 19.2% increase in infection frequency over a 7-year period (Figure 2). There was no significant difference between the three years for infection frequency based on Z-tests [Z-test for equal proportions: Z (’06, ’09) = 0.756, P = 0.447; Z (’09, ’13) = 0.720, P = 0.471; Z (’06, ’13) = 1.497, P = 0.133]. The northernmost population sampled (Georgia) had an 8.1% decrease in frequency over 3 years (Figure 2). Like the Louisiana samples, the Georgia population showed no significant difference between the years for *Wolbachia* infection [Z-test for equal proportions: Z (’13, ’16) = 1.457, P = 0.144]. The 2006 Louisiana test for equal proportions of *Wolbachia* infected and uninfected individuals was significant, but the other two years (2009 and 2013) showed no significant difference between infected and uninfected (Table 1). Both years in Georgia (2013 and 2016) showed a significant difference from equal proportions of infected and uninfected individuals (Table 2).

For the Louisiana samples, the 2009 *Wolbachia* negative samples had the least amount of gene diversity in the population, while the 2006 positive population had the highest gene diversity (Table 3). There is a decrease in Hd over time, though this decrease is not significant (Table 3). In the Georgia samples, the 2013 positive samples have the lowest amount of gene diversity (Table 3). However, gene diversity increased in the 2016 positive samples (Table 3).

For the Florida population, there is only one time point available, however, there is no difference in Hd between the positive and negative samples for this population (Table 3). There was no indication of differences in haplotype diversity estimates among locations sampled despite differences in *Wolbachia* frequency (Figure 2).

The π value for Louisiana indicated that there is little heterogeneity between infection status, as well as years for this population (Table 3). The largest variance can be seen in the 2006 *Wolbachia* positive samples, while the 2013 positive samples were the least variable (Table 3). There is also no difference in nucleotide diversity for the Georgia and the Florida populations (Tables 3). Both populations have low π values for all years, as well as infection status, meaning that there is no difference in nucleotide diversity based on these two factors (Tables 3).

For the Louisiana samples, the 2006 positive samples had the highest amount of variation in that it has a higher mutation rate per sequence (Θw = 13.113), while all other Louisiana samples were relatively similar regardless of time point and infection status (Θw =6.828-3.616) showing no difference in mutation rate (Table 3). Θw for Georgia ranged from 3.535 to 7.883, with the 2016 positive sample having the highest mutation rate and the 2013 positive samples having the lowest (Table 3). The Florida samples showed a large difference between infected and uninfected in mutation rate, with the uninfected samples having a larger rate (Table 3).

The total population consists of primarily four main haplotypes: H01 (11%), H02 (21%), H03 (26%), and H04 (17%) (Figure 4). No single haplotype was associated with Wolbachia infection. The four main haplotypes had the following *Wolbachia* infection frequencies: H01 (19%), H02 (37%), H03 (48%), and H04 (37%). A Bayesian analysis suggests that there are four main clades of haplotypes in the gene tree. Each of the four main haplotypes are distributed in

different clades (Figure 3). *Wolbachia* infection and absence was also distributed through all clades (Figure 3).

DISCUSSION

Latrodectus geometricus is a recently introduced species that has been found to harbor *Wolbachia* (Arrington 2014). The distribution of *Wolbachia* infection frequency among Southeastern brown widows ranged from 9.2% to 90%, which is consistent with frequencies described by Arrington (2014). There is also a potential geographic pattern on higher *Wolbachia* infection frequency with a decreasing latitude as the northern populations tend to have a lower *Wolbachia* infection frequency relative to the more southern samples This range of infection frequency in different populations allows for a unique opportunity to investigate the potential relationship between *Wolbachia* and mitochondrial haplotype diversity.

With the wide range in *Wolbachia* frequency across different populations (9% to 90%), it is clear that the host-endosymbiont relationship in this case is not obligate. An obligate relationship between *Wolbachia* and host has been described in several species, including infected parasitic wasps where infection is necessary for oogenesis (Dedeine *et al.* 2001, Stahlhut *et al*. 2006), in filarial nematodes where infection is needed for development (McGarry *et al*. 2004), and in bed-bugs where infection is essential for growth and reproduction (Hosokawa 2010). Fixation of *Wolbachia* in a population can also be driven by strong reproductive manipulations of the host by the bacteria (Zug and Hammerstein 2015

Mitochondrial DNA variation does not differ between *Wolbachia* infected individuals when compared to uninfected individuals within and among brown widow populations. Strong linkage between mtDNA and *Wolbachia* can be established when *Wolbachia* causes strong

fitness effects on the host (Müller *et al*. 2012). This linkage would result in selective sweeps/hitchhiking resulting in lower mtDNA variation in *Wolbachia* infected populations compared to *Wolbachia* uninfected populations (Shoemaker *et al*. 2000). *Wolbachia* associated selective sweeps of mitochondria have been seen in fig wasp species (Sun *et al*. 2011), blow flies (Baudry *et al*. 2003), mosquitos (Kambhampati *et al*. 1992), and *Drosophila* (Ballard *et al*. 2006). In brown widow populations, mtDNA variation data does not indicate a recent selective sweep suggesting a lack of linkage between the bacterium and mitochondria in brown widow populations. The absence of a hitchhiking effect between the endosymbiont and mitochondria can be explained by a *Wolbachia* infection that has weak or no effect on host fitness (Müller *et al*. 2012). This absence of an association between *Wolbachia* and mtDNA has also been seen in *Drosophila willistoni, Drosophila yakuba,* and *Solenopsis invicta* (Müller *et al.* 2012). This weak association can be due to several factors including: the infection being recent where the full selective sweep has not yet occurred, the infection being lost in the populations, reproductive parasitism occurring at low levels or is absent, or paternal leakage of mitochondria (Müller *et al.* 2012). A recent infection seems unlikely, as high levels of horizontal transfer would be necessary to result in the haplotype-*Wolbachia* pattern seen in the gene tree. (Figure 3). While a paternal leakage is plausible, there was no indication of individuals having heteroplasmy in the sequencing results, which would appear as two competing peaks when sequencing (Müller *et al.* 2012). However, our system tends to point to mtDNA variation and *Wolbachia* in brown widow population are most consistent with some level of *Wolbachia* loss over time often associated with weak or low levels of reproductive parasitism.

The absence of association between mtDNA variation and *Wolbachia* infection in introduced brown widow populations is unlikely to be the consequence of a recent infection

where a selective sweep has not yet occurred. If the *Wolbachia* infection in brown widows is a recent event, a single mtDNA haplotype or a small number of related haplotypes would be predicted to be associated with *Wolbachia,* while a diversity of haplotypes would be found among uninfected individuals (Müller *et al.* 2012). *Wolbachia* infection was present with an equal diversity of haplotypes in infected and uninfected individuals, (Figure 3). A correlated change in mtDNA diversity with changes in *Wolbachia* frequency in a population over time would also be expected if this was a recent infection and strong fitness consequences for the host. The frequency of infection did increase by 19.2% in the Louisiana samples over a 7-year period, while the Georgia samples showed an 8.1% decrease over 3 years, yet neither of these were significant changes (Figure 2; Tables 1 and 2). In both cases, there was no indication of a correlated change in mtDNA diversity or a strong association with a single haplotype.

Populations with variable *Wolbachia* infection frequency lacking correlation with mtDNA variation can be explained by recent infection if horizontal transmission occurs. Charlat et al. (2004) referred to this as the never infected hypothesis, where there may be cytoplasmic lineages that have never been infected (Charlat *et al*. 2004). Under the never infected hypothesis, there can be two scenarios that would result in the low infection frequency (Charlat *et al.* 2004). One scenario involves recent horizontal transmissions of a non-CI inducing *Wolbachia* behaving like a neutral trait. Low infection frequencies occur because horizontal transfer and drift are in temporary equilibrium, and fixation of the infection is never reached (Charlat *et al.* 2004). The second scenario also includes horizontal transmission and drift, where CI expression was secondarily lost. Some of the populations had a fixed infection, while others were never infected, and interbreeding of the two followed (Charlat *et al*. 2004).

A second explanation of variable *Wolbachia* infection frequency lacking correlation with mtDNA variation is the once infected hypothesis. In this hypothesis, the association is old between host and symbiont and transmission has become imperfect from originally infected frequencies (Charlat *et al.* 2004). The once infected hypothesis suggests that the host-symbiont relationship is ancestral and maternal transmission has become imperfect, resulting in loss of infection (Charlat *et al.* 2004). Ancestral expression of cytoplasmic incompatibility (CI) followed by secondary loss or reduction in CI can also be inferred from this hypothesis (Charlat *et al.* 2004). This hypothesis cannot be ruled out in brown widow-*Wolbachia* interaction (this hypothesis is partially assessed in chapter 2). *Wolbachia* infection being ancestral with subsequent loss is consistent with the mapping of *Wolbachia* infection frequency onto the mtDNA gene tree (Figure 3). Most of the population (83%) carries one of four haplotypes: H01, H02, H03, and H04, with these four haplotype strains being associated with *Wolbachia* at various infection frequencies less than 1 (Figure 3). These four major haplotypes are not closely related and fall into the four major clades with less recurrent haplotypes within each clade. Many of the less common haplotypes also occur with *Wolbachia* infection and many do not. This pattern is consistent with a scenario where, historically, *Wolbachia* may have been fixed or at high frequency in the population, but over time selection for the infection weakened. This weakening could then have allowed for random losses of mtDNA haplotypes associated with infection and allowing for newer haplotypes to begin arise. If the mtDNA phylogeny had shown several haplotypes with high infection frequency related in a single lineage, a new infection or ongoing selective sweep would have been inferred. This pattern is seen in fig wasps where infected and uninfected mtDNA haplotypes separate into two major clades (Xiao 2011). Over generations, *Wolbachia* associated haplotypes increase in frequency in the population and

haplotypes that are not associated with the bacteria eventually become less common if reproductive manipulations are strong. Strong cytoplasmic incompatibility for example would drive rapid increase in infection frequency, as infected females have twice the chance that uninfected females do at finding a mate to produce viable offspring (Charlat *et al.* 2004). The pattern of one related lineage associated with infection was not see in the brown widow populations studied here. This suggests that that the infection could be becoming lost through the weakening of strong selective pressures, such as CI and incomplete transmission.

The distribution of infection frequency in Southeastern US populations of brown widows shows a trend of decreasing infection rate with increasing latitude (Figure 1). This distribution could be the consequence of: multiple founder events during introduction (Arrington 2014), the loss of infection as the spider enters novel environments caused by differing selective pressures on infection status (Shoemaker *et al*. 2000, Tsutsui *et al*. 2003, Reuter *et al*. 2005), or environmental differences that can cause host benefits to be lost, thus removing the bacterium from the population as the infection becomes either neutral or costly to the host (Stouthamer *et al*. 1999, Charlat *et al*. 2004). The pattern of increase in the Louisiana population infection frequency by 19.2% over a 7-year period, while the Georgia populations decreased by 8.1% over 3 years is consistent with the latitudinal gradient described by Arrington (2004). There is higher infection frequency in lower latitudes and a lower in infection frequency in higher latitudes. These two observations of south to north decrease in infection warrants the hypothesis that as the spider expands to novel environments, there is a loss of infection due to different selective pressures on infection status (Shoemaker *et al*. 2000, Tsutsui *et al*. 2003, Reuter *et al*. 2005). In populations found in lower latitudes, effects of infection may be beneficial for survival in a warmer environment, but as the spider expands towards the north and cooler climates, these

benefits might become neutral or even costly. *Wolbachia* has been found to provide a heat-tolerance benefit in pea aphids when compared to uninfected samples. (Chen *et al*. 1997, 2000, Russell and Moran 2006). Infection was also found to increase fecundity when aphids were under heat-stress (Montllor *et al*. 2002). Temperature has also been found to affect maternal transmission, microbe replication rate, and influence microbe-density (Douglas 1994, Mouton 2006), which could lead to a change in phenotype. Temperature effects on the host and endosymbiont relationship should be further investigated in brown widows.

In this study, I have determined that there is no linkage between *Wolbachia* infection and mitochondrial DNA variation in brown widows. The absence of linkage between mitochondrial haplotypes and *Wolbachia* supports the hypothesis that the infection is old and is being lost, suggesting that host fitness effects may be weak. Support for this hypothesis would be strengthened by testing the CI strength in this system. A trend of decreasing frequency in the North and increasing frequencies in the south suggests that the distribution is not random. Additional studies of symbiont and environmental fitness effects, including bacterial load differences, fitness, and temperature effects are needed.

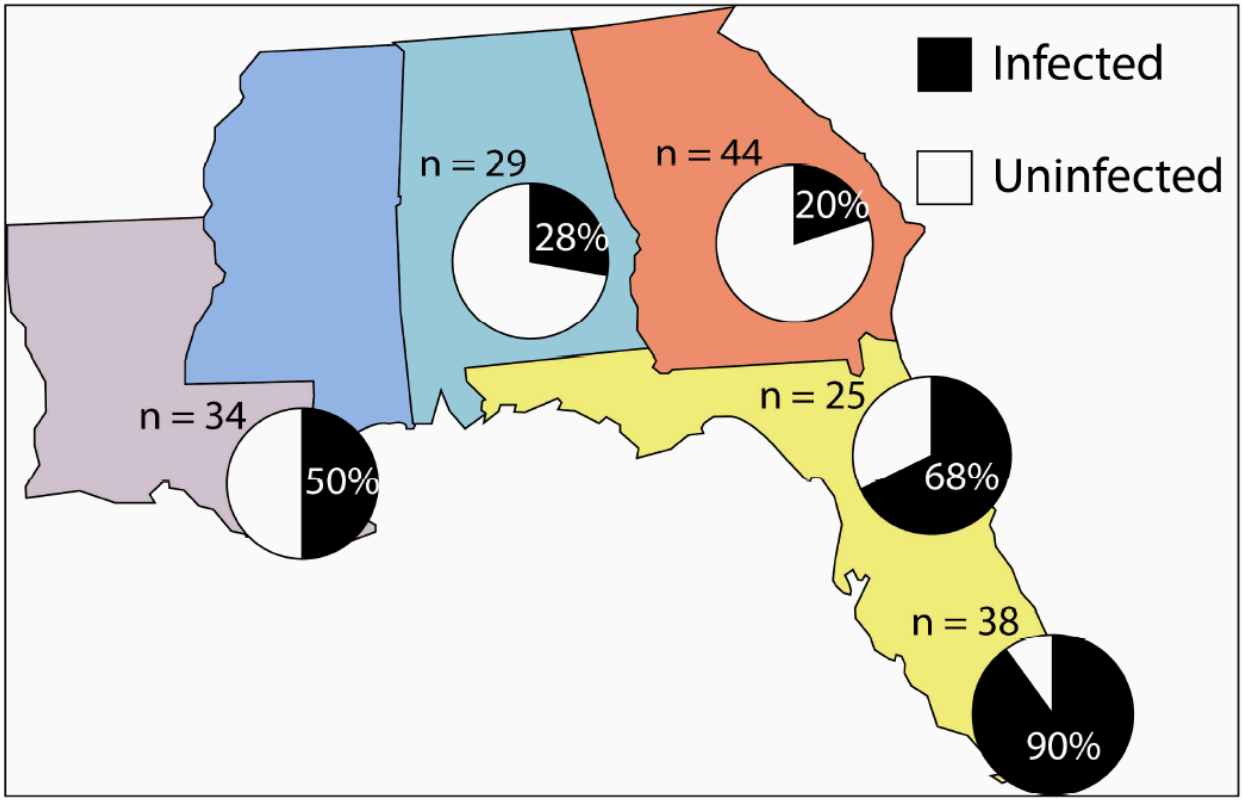

Figure 1. Prevalence of *Wolbachia* in Brown Widow Spiders (*Latrodectus geometricus*) from five Southeastern populations (Arrington 2014)

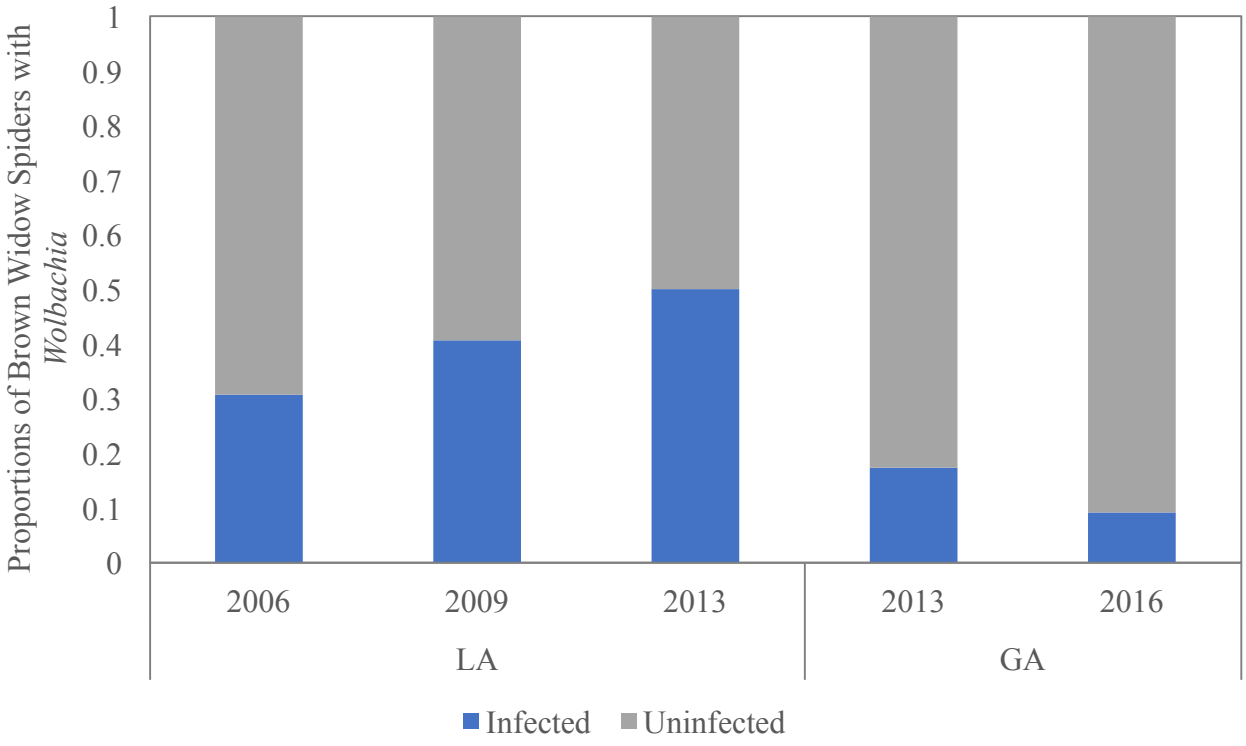

Figure 2. Proportions of brown widow spiders infected with *Wolbachia* in Louisiana and Georgia over time.

Table 1. Chi-square test of equal frequencies by year, in Louisiana.

	2006	2009	2013
Infected	8	11	17
Uninfected	18	16	17
χ^2	5.31	0.93	0
P-value	0.021*	0.33	1

Table 2. Chi-square test of equal frequencies by year, in Georgia.

	2013	2016
Infected	17	6
Uninfected	81	59
Chi^2	45.43	50.089
P-value	<0.0001*	<0.0001*

Table 3. Genetic diversity estimates of mitochondrial cytochrome oxidase I gene in brown widow populations

Population	Haplotype (gene) Diversity, Hd	Nucleotide Diversity, ∏	Θw	n
LA ‘06 Positive	0.893 ± 0.111 S.D.	0.02338	13.113	8
LA ‘06 Negative	0.894 ± 0.078 S.D.	0.01265	5.961	12
LA ‘09 Positive	0.709 ± 0.237 S.D.	0.01163	6.828	11
LA ‘09 Negative	0.592 ± 0.122 S.D.	0.00956	5.425	16
LA ‘13 Positive	0.658 ± 0.108 S.D.	0.0082	3.616	16
LA ‘13 Negative	0.705 ± 0.074 S.D.	0.01058	6.766	15
GA ‘13 Positive	0.467 ± 0.132 S.D.	0	3.535	10
GA ‘13 Negative	0.786 ± 0.028 S.D.	0.0126	6.722	57
GA ‘16 Positive	0.733 ± 0.155 S.D.	0.01399	7.883	6
GA ‘16 Negative	0.794 ± 0.031 S.D.	0.01335	5.093	63
FL ‘13 Positive	0.708 ± 0.031 S.D.	0.01366	3.786	30
FL ‘13 Negative	0.667 ± 0.314 S.D.	0.01763	10.667	3

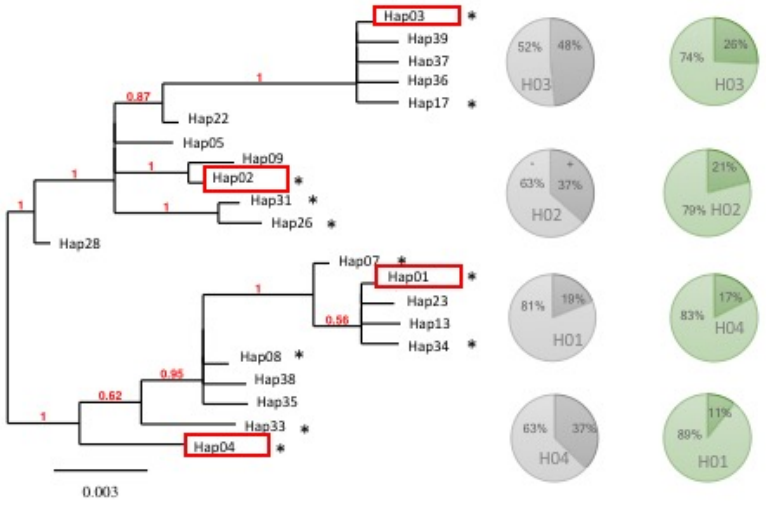

Figure 3. Phylogenetic analysis of mitochondrial haplotypes. The phylogeny shows posterior probabilities from a Bayesian analysis. The charts illustrate the four predominant haplotypes, which are outlined in red: Haplotype 01, Haplotype 02, Haplotype 03, and Haplotype 04. The left pie charts shown in gray gives *Wolbachia* infection frequency for each haplotype, with dark gray representing the infected samples and the light gray representing uninfected samples. The right pie charts in green show the frequency of the specific haplotype within the population, with dark green representing that specific haplotype and light green representing the rest of the population not found with that haplotype. The haplotypes with asterisks denote haplotypes associated with infection.

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CHAPTER 2

Consequences of *Wolbachia* Infection on Cytoplasmic Incompatibility in *Latrodectus geometricus*

ABSTRACT

Wolbachia are maternally inherited bacteria that have been found to manipulate host reproduction through a variety of mechanisms including: feminization, parthenogenesis, male-killing, and cytoplasmic incompatibility. Cytoplasmic incompatibility (CI), when the sperm from an infected individual and the egg from an uninfected individual are unable to produce viable offspring, can be expressed at different intensities. *Wolbachia* was recently found in introduced populations of the brown widow spider, *Latrodectus geometricus*, in the Southeastern US. In this system, infection frequencies vary among introduced populations. With the potential manipulations, understanding the biology of the spider is limited until the relationship of the endosymbiont and spider is better understood or resolved. The goal of this study is to determine if *Wolbachia* induces CI in the brown widow through controlled breeding crosses. It was found that *Wolbachia pipientis* induced partial CI in the brown widow, as hatching frequency was reduced by 35.5% when infected males were mated with uninfected females, compared to that of mating of uninfected females and uninfected males. Partial CI indicates *Wolbachia* virulence may be reduced over time thus allowing for a decrease in selective pressure against uninfected spiders and maintenance of variable infection frequencies among introduced populations.

INTRODUCTION

Invertebrates, particularly Arthropoda, have been found to harbor bacterial endosymbionts (Zchori-Fein and Bourtzis 2011). Host/endosymbiont interactions can include: mutualism, a symbiotic relationship where both benefit; commensalism, a symbiotic relationship where one benefits and the other is neither benefitted or harmed; or parasitism, a symbiotic relationship where one benefits at the expense of the other (Werren 2008). The bacterial endosymbiont, *Wolbachia pipientis*, can be a reproductive parasite of its arthropod hosts (Werren 2008). *Wolbachia* has intrigued scientists as it fundamentally violates the view that heritable symbionts are mutualists (Zug and Hammerstein 2015). *Wolbachia* is maternally, vertically inherited and has been found in the cytoplasm of somatic and germ line cells within the host (Shoemaker *et al.* 2002, Zug and Hammerstein 2015).

Wolbachia is extremely common in arthropods. Estimates indicate that over 65% of insect species possess this endosymbiont (Werren *et al*. 1997, Werren and Windsor 2000, Jiggins *et al.* 2001, Hilgenboecker *et al.* 2008). A taxonomically diverse survey of 63 arthropod species showed a 76% infection frequency (Jeyaprakash and Hoy 2000). Rowley *et al.* (2004) found *Wolbachia* infections in 7 of 10 spider families sampled.

Wolbachia is predominantly transmitted vertically and can cause a decrease in host fitness by harmfully manipulating host reproduction for their own benefit (Zug and Hammerstein 2015). This decrease in fitness may be offset by a potential giving of nutrients to the host to allow for retention of the bacteria (Hosokawa *et al*. 2009). *Wolbachia* has acquired four different mechanisms to induce these manipulations: male feminization, parthenogenesis induction, male killing, and cytoplasmic incompatibility (CI) (Werren 2008). Male feminization is when genetic males develop as functional females (Werren 1997, 2008). Parthenogenesis is the development

of unfertilized eggs laid by virgin haplodiploid females (Werren 2008, Russell and Stouthamer 2011). Male killing is when male embryos laid by an infected mother do not develop (Werren 2008, Sakamoto 2011). Cytoplasmic incompatibility results in a lack of embryonic development when infected males mate with uninfected females or females infected by a different strain of the symbiont (Turelli 1994, Hoffman and Turelli 1997). These reproductive manipulations allow the bacterium to increase their fitness by increasing the number of infected females in forthcoming generations and therefore increasing their frequency in host populations (Werren 2008, Zug and Hammerstein 2015).

Out of the reproductive manipulations induced by *Wolbachia*, cytoplasmic incompatibility (CI) is the most common (Werren 2008). *Wolbachia* can render sperm nonfunctional with the modification being rescued if the eggs are infected with the same strain. If the *Wolbachia* is not present in the egg, the sperm will remain modified and the embryo will not initiate development (Werren 1997). Modification of the sperm most likely occurs at an early stage of spermatogenesis, as the bacteria is shed from the sperm into cytoplasmic waste (Bressac and Rousset 1993). Werren (1997) proposed a "modification and rescue model" where *mod* modifies the sperm and *resc* occurs in the egg where it restores the sperm functionality (Poinsot *et al.* 2003). Three models have been proposed to explain how *mod resc* occurs in a host: i) the "lock-and-key" model, where the *mod* is caused by a "lock" that is produced by the bacteria and binds to the paternal nucleus and must be unlocked by the "key" present in an infected egg to remove the lock and produce viable offspring (Poinsot *et al.* 2003); ii) the "sink" model, whereby *Wolbachia* in an infected male removes proteins associated with chromosomes (*mod*) and gives them back (*resc*) after being fertilized by an infected female (Kose and Karr 1995, Werren 1997, Poinsot *et al.* 2003); and iii) and the "slow-motion" model, in which infected

paternal chromosomes are delayed entry into mitosis (*mod*) and *resc* is caused by a similar modification in infected mothers that allows a restoration of a synchronous cycle between the two (Reed and Werren 1995, Callaini *et al.* 1997, Poinsot *et al.* 2003).

CI can occur at different intensities, or percentages of embryos that do not develop, in crosses between infected males and uninfected females (Charlat *et al.* 2004, Frank 1998). The level of CI can play a large role on the rate of infection increase in a population. For example, if the CI is strong within the population, then the frequency of infection will increase in the population or stay at fixation (Frank 1998). However, if CI is very weak, the frequency of infection can decrease from 100% to a lower rate due to invading host factors, like the general health or nutritional state of the organism (Frank 1998). There are many factors that can influence the expression of CI, including the strain of bacteria, the host genotype, and the density of the bacteria (Werren 1997). *Wolbachia* infected females can have lower fecundity than their uninfected counterparts, but the infected females achieve a reproductive advantage, as they generally produce viable offspring with both infected and uninfected males (Hoffmann *et al.* 1990, Turelli 1994). If CI is strong, infected females have a higher relative fitness than uninfected females. *Wolbachia* will consequently spread rapidly and increase to fixation within the population (Hurst 1991). However, Prout (1994) suggested that variants of *Wolbachia* may be able to increase the fecundity of infected females, even if they increase the compatibility of a cross between infected males and uninfected females, resulting in different CI intensities. CI has been found to have several variances in laboratory and natural populations. Hoffmann *et al.* (1990) found that the severity of CI is generally greater in a lab setting and that infected females may exhibit reduced fecundity in the lab when compared to natural populations. Some of the variation in CI levels may be controlled by host genes. Hoffmann (1988) found that *Wolbachia*

that caused high levels of CI in *D. simulans* produced low levels of CI when transferred to *D. melanogaster*. Dosage of *Wolbachia*, or bacterial loads, may also explain difference in CI levels (Breeuwer and Werren 1993). Boyle *et al.* (1993) noted that higher levels of CI were associated with higher levels of bacteria in the eggs.

Wolbachia variants have been divided into eight supergroups (A-H), with super groups C and D commonly found in filarial nematodes and the other six supergroups found predominantly in arthropods. *Wolbachia* is known to have different variants in various supergroups that may affect CI levels, with related forms of *Wolbachia* producing different host compatibility types (O'Neill *et al.* 1992, Rousset *et al.* 1992). Supergroups A and B are the most common in arthropods (Baldo *et al.* 2007, Werren *et al.* 2008). Supergroup F is a unique supergroup having been found in termites (Lo and Evans 2007), filarial nematodes (Casiraghi *et al.* 2005), bed bugs (Hosakawa 2010), bush crickets (Panaram and Marshall 2007), lice (Covacin and Barker 2007), and Southern African scorpions (Baldo *et al.* 2007). The effects of supergroup F on their hosts are largely unknown, except Hosakawa (2010) found that *Wolbachia* aided in nutrient acquisition of vitamin B that promoted egg development in bed bugs.

The brown widow spider (*L. geometricus*) is a non-native species that has been found to harbor *Wolbachia* (Arrington 2014). The brown widow is thought to have originated in South Africa and has now been introduced to all continents except Antarctica, through human introduction (Garb *et al.* 2004, Brown *et al.* 2008). In the 20th century, the brown widow was introduced into the southern peninsula of Florida and has rapidly expanded its range in the Southeastern US (Brown *et al.* 2008). The spider can now be found as far north as South Carolina and as far west as Texas (Brown *et al.* 2008) and has been introduced into Hawaii (Pinter 1980) and California (Garb *et al.* 2004). A unique strain of supergroup F was discovered

in the brown widow spider, *Latrodectus geometricus*, when compared to known strains, including the strain found in its relative *L. mactans* (J.S. Harrison unpublished data). The relationship between *Wolbachia* and *L. geometricus* is not obligate as infection frequency ranged from 20% to 90% in tested populations (Arrington 2014). One explanation for *Wolbachia* not being fixed in populations is a facultative or neutral relationship between the symbiont and host (Arrington 2014). *Wolbachia* may have been previously fixed within the sampled populations, however, through the loss of strong CI, the bacteria frequency may be lost with associated neutral effects, such as drift or competition (Reuter *et al.* 2005). Another explanation to fit the distribution pattern are recent founder events from multiple source populations that differ in infection status and limited gene flow among Southeastern US locations (Arrington 2014). The current effect of *Wolbachia* on *L. geometricus* is unknown. A recent study concluded that *Wolbachia* does not influence clutch sex ratio, egg number, egg size, egg mass, or development time in *L. geometricus* (Arrington 2014). These results eliminate the possibilities of male feminization, parthenogenesis, or male killing as possible reproductive manipulations of *Wolbachia* on the brown widow spider. CI has not yet been tested in this species.

The aim of this study is to determine if *Wolbachia pipientis* induces CI in the brown widow spider. I ask the following question: Does a cross between uninfected females and *Wolbachia* infected males induce complete embryonic death? I hypothesize that a cross between uninfected females and *Wolbachia* infected males will induce CI and result in embryo death. However, complete cytoplasmic incompatibility (100% embryo death) will not be seen, as the infection is not fixed within the brown widow populations (Chapter 1, Frank 1998, Arrington 2014).

METHODS

Testing for Wolbachia

Female brown widow spiders were collected from the greater Statesboro, GA area. Females were found with egg sacs or laid egg sacs once in the lab. A whole leg was taken from the mother for DNA extraction and Polymerase Chain Reaction (PCR). The DNA extraction was done using the ZR Genomic DNA™- Tissue Microprep kit (Zymo Research). The *Wolbachia* specific primer for the fructose-bisphosphate aldolase gene (FbpA) was chosen for PCR, as this primer has been found to amplify in all known strains of *Wolbachia* (Arrington 2014, Simões 2011, Vanthournout *et al.* 2001). The PCR protocol for the FbpA primers followed Simões *et al.* (2011). The PCR products were run on a 1% agarose gel. *Wolbachia* infection status was determined by the presence of PCR product for the FbpA gene.

Rearing spiders for breeding

All spider rearing was carried out in an incubator set at 27°C, 50-60% humidity, and a 12-hour light-dark cycle. Spiders used for mating were raised from egg sacs laid in lab, so virgin females and males of known ancestry could be used to remove maternal effects other than *Wolbachia* presence/absence. The egg sacs were opened, upon arrival to lab or once they were laid, and the offspring were allowed to develop in a petri dish. After the first molt, spiderlings were placed into individual cages. The immature spiders were fed 3-4 wingless drosophila twice a week. Sex was determined in the 3rd and 4th instar by using differences in pedipalp size and looking for the swelling of the palps in males (Mahmoudi *et al.* 2008, Kaston 1970). Once sex was determined, the females were fed small mealworms, while the males were continued to be fed drosophila due to ensure that adequate nutrition was being given. Crosses were determined based on *Wolbachia* infection status of the mothers. Males used in the study were not able to be

tested for infection status without being sacrificed. I assumed all males of infected mothers were infected based on a survey of infection of 96 offspring from each of four infected mothers. In each case, *Wolbachia* transmission rates from mother to offspring was 100% (J.S. Harrison unpublished data).

Cytoplasmic Incompatibility Assay

Four crossing treatments were completed, with "*w"* meaning that an individual is positive for *Wolbachia*: ♀ x ♂, *w*♀ x ♂, *w*♀ x *w*♂, ♀ x *w*♂. A total of 15 lab- reared virgin females (4 infected, 11 uninfected) were mated with unrelated lab reared virgin males (7 infected, 8 uninfected). The control crosses of ♀ x ♂ had five breeding pair replicates, *w*♀ x ♂ had three breeding pairs, and *w*♀ x *w*♂ had only one breeding pair. The experimental cross of ♀ x *w*♂ had six breeding pairs. Once females were mature enough to breed, as determined by size and number of molts, they were fed a mealworm in hopes of increasing the survival of a male and a predetermined male was placed in the cage. The male was left in the cage for three days or until the male had been killed. The males who were removed were observed under a dissecting microscope to determine if their reproductive organs were intact. If they were not, there was a high probability that a successful mating had occurred.

Collecting egg and hatching numbers

The day each female began to produce egg sacs was recorded, with the sac being removed from the cage after two days. The egg sac was placed in a petri dish, opened using forceps, and the total number of eggs counted. The number of unhatched eggs counted were counted every two days until the number of unhatched eggs did not change for a two-week period. Females are able to produce multiple egg sacs from a single mating; therefore, each time a female laid an egg sac, egg sac number, egg counts, hatching date, and the number of

unhatched eggs were recorded. Averages of all clutches from individual breeding pairs were calculated and the data was analyzed with a one-way ANOVA. The data was tested for normality with a Shapiro-Wilk test. The data was log-transformed to attain normality. A Tukey HSD post hoc test was conducted to determine if there was a statistical significance between groups.

RESULTS

The average hatching rate of egg clutches produced from *Wolbachia* uninfected females mated with infected males (♀ x *w*♂) was 55.1% ± 12.03%, which was a reduction of 35.5% when compared to the hatching rates of ♀ x ♂ crosses (90.6% ± 4.67% hatching rate) (Figure 4). Additionally, there was a 33.1% reduction when ♀ x *w*♂ was compared to the 88.2% ± 7.19% hatching rate of *w* ♀ x ♂ (Figure 4). There was a significant difference between the hatching numbers of the two control groups of ♀ x ♂ and *w*♀ x ♂ when compared to the experimental group of ♀ x *w*♂. (P= 0.059, DF= 13) after the data was log transformed and a Tukey test conducted (Table 4). The crosses of infected males with infected females were removed from the analysis due to the low sample size (n = 1). Large variation can also be seen within clutches of the same breeding pairs (Figure 5).

DISCUSSION

Wolbachia infection in *L. geometricus* appears to induce CI in crosses between non-infected females and infected males. This CI is partial, as there is not complete embryotic death. Partial CI has also been seen in the planthopper, *Sogatella furcifera* (Noda *et al.* 2000). In *S. furcifera,* partial CI is correlated with a lower density of *Wolbachia* when compared to another planthopper, *Laodelphax striatellus*, which had high levels of CI (Noda *et al*. 2000). A certain threshold of bacterial density may be required for modification of paternal genetic material (Noda *et al.* 2000). The level of CI seen in the brown widow may be a consequence of low or

variable *Wolbachia* densities compared to arthropod species with total CI. There was considerable variation in hatching number among replicates of the ♀ x *w*♂ cross. This may indicate that high levels of variation exist in *Wolbachia* density among infected individuals, which could be tested in future experiments.

Temperature and male age are known to influence CI levels (Clancy and Hoffman 1998, Singh *et al.* 1976). The spiders were kept in an incubator at 27°C, which allowed us to mimic natural conditions for the Southeastern United States (Arrington 2014). This natural condition mimicry appears to enhance the amount of eggs laid by a female (Arrington 2014). However, Clancy and Hoffmann (1998) found that in *D. simulans*, higher temperatures negatively impacted the intensity of CI, as *Wolbachia* density in embryos was reduced with exposure to 25 °C when compared with a 19 °C treatment. Arrington (2014) found a decrease in infection frequency as latitude increased. This latitudinal decline in infection frequency could indicate that there are different selective pressures on infection with one possibility being CI intensity (Shoemaker *et al.* 2000, Tsutsui *et al.* 2003, Reuter *et al.* 2005). In South Florida, the infection may be beneficial for temperature effects on CI, however, in novel higher latitude environments, the effects may be weakened (Chen *et al.* 1997, 2000, Russell and Moran 2006,).

The male age for the experiment was not kept constant, due to the limited numbers of infected virgin males available to breed, but there is no indication of systematic bias as the ages were varied for both infected and uninfected males. The differences in CI within the experimental group of ♀ x *w*♂ (Figure 5) could be related to the differences in male age. Older males were found to have reduced amounts of CI in *D. simulans* (Hoffmann *et al.* 1986), due to a decrease in the amount of *Wolbachia*- infected sperm (Bressac and Rousset 1993). This decrease in *Wolbachia* results in a decrease in the modification of sperm (Noda *et al.* 2000), which could

allow for uninfected females to produce viable offspring with older infected males. The combined effects of temperature and male age may explain the partial CI seen in *L. geometricus*.

Partial CI can be a consequence of reduced virulence of *Wolbachia* on the host over time (Hoffmann and Turelli 1997). This reduction in selective pressures often leads to reductions in *Wolbachia* frequency within the population. The variation in *Wolbachia* infection frequency in Southeastern US *L. geometricus* populations is consistent with low virulence and selective pressures (Arrington 2014). The absence of = linkage between mitochondrial DNA and *Wolbachia* infection demonstrated in chapter 1 would also result from weak host manipulation. With low intensity of CI in *L. geometricus,* the selective forces driving increased frequency of *Wolbachia* in a population is greatly reduced (Hoffman and Turelli 1997). Therefore, the frequency of *Wolbachia* would be driven primarily by drift after introduction into novel habitats (Reuter *et al.* 2005). *Wolbachia* frequency can drop below a certain threshold, determined by the effect of infection on host fertility and the rate of vertical transmission, and the probability of bacterial loss increases (Caspari and Watson 1959, Hoffmann and Turelli 1997). Founder effects, in combination with weak CI, could have reduced the infection below the threshold and could have allowed the maintenance of low infection frequency (Charlat 2004, Reuter *et al.* 2005).

Finally, there may be a clutch affect occurring in the experimental crosses of ♀ x *w*♂. Hatching rate variation is high within the experimental breeding crosses (♀ x *w*♂) (Figure 5). There is also high variation in hatching number between successive clutches produced by each female in the ♀ x *w*♂ crosses. This could be due to the maternal effects such as the mother putting more energy into certain clutches, thus overriding the effects of *Wolbachia*. It could also be explained through another mechanism that would cause a dilution of *Wolbachia*, which could explain widespread *Wolbachia* throughout the body seen in chapter 3. The mechanisms driving

CI intensity variation between successive egg clutches need to be investigated in brown widows. More studies on clutch affect would need to be conducted to further understand CI in the brown widow, including determining if there is a difference in the dilution of bacteria in each egg sac or if different individuals in each clutch and what this mechanism consists of.

This study provides evidence that *Wolbachia* infection in *L. geometricus* causes only weak CI. Bacterial density, temperature, and male age can affect the levels of CI expressed in this organism and should be tested as potential mechanisms. Clutch effects, in which the same breeding cross of ♀ x *w*♂ had variation in hatching number between egg sacs, suggest that maternal effects can influence the intensity of CI. Partial CI in *L. geometricus* is likely a contributing factor to the variable frequency of *Wolbachia* infection among introduced Southeastern US populations rather than this being a new infection in introduced populations (Charlat 2004).

Table 4: Analysis of Variance of average hatching numbers (log transformed) for the crosses of ♀ x ♂, *w*♀ x ♂, and ♀ x *w*♂.

Analysis of Variance					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**	**Prob > F**
Infection Status	2	0.5929570	0.296478	3.9578	0.051*
Error	11	0.8240009	0.074909		
C. Total	13	1.4169579			

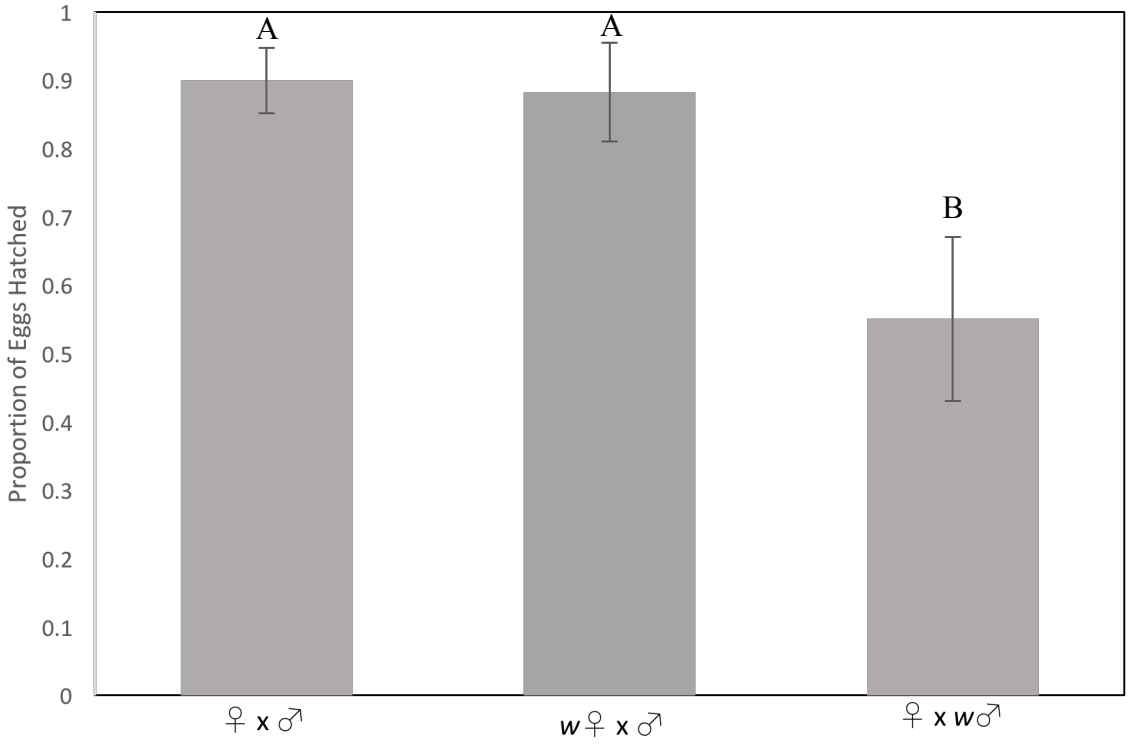

Figure 4: Average proportion of eggs hatched (± s.e.) for the crosses of ♀ x ♂, *w*♀ x ♂, and ♀ x *w*♂. Hatching numbers were lower in the cross of ♀ x *w*♂ when compared to those of ♀ x ♂ and *w*♀ x ♂ crosses.

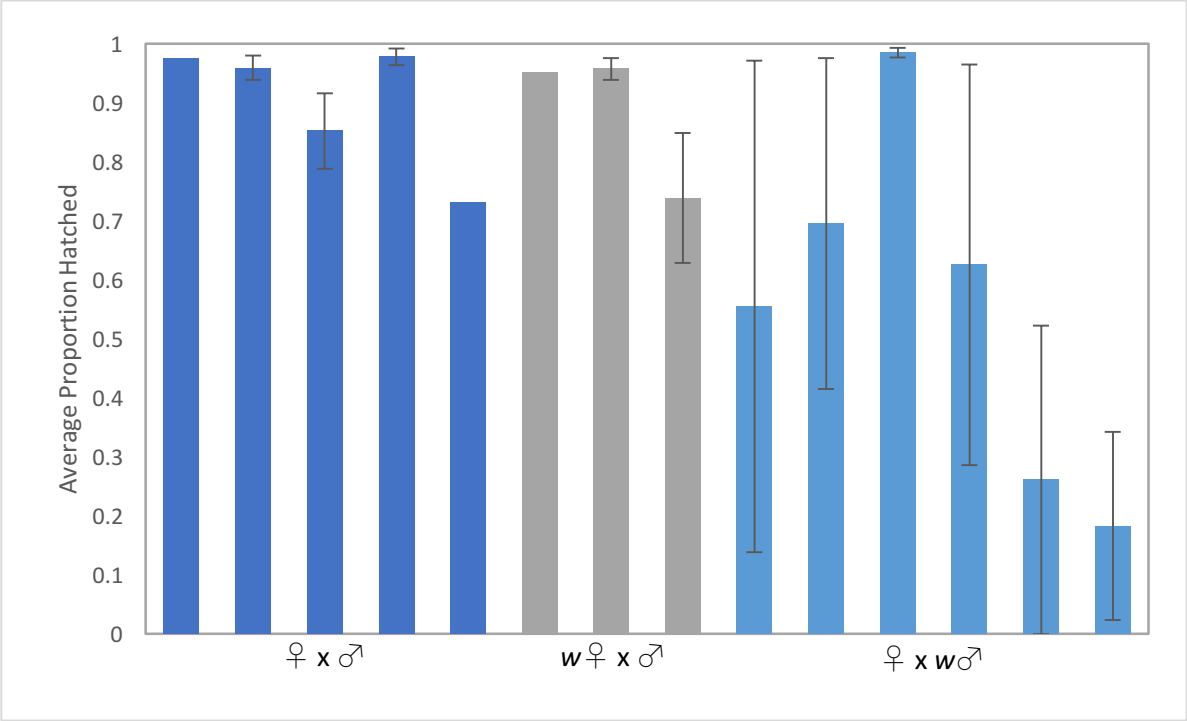

Figure 5. Average proportion of eggs hatched (± s.e.); each bar represents a single female producing multiple clutches. Hatching numbers varied for each cross, with the negative female and positive male crosses ♀ x *w*♂ having the largest variability.

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CHAPTER 3

Wolbachia Infection Localization and Bacterial Load Among Three Body Regions of *Latrodectus geometricus*

ABSTRACT

The bacterial endosymbiont *Wolbachia pipientis* is a maternally inherited cytoplasmic parasite known to use reproductive manipulations on its host to increase its own fitness and frequency in host populations. *Wolbachia* has been found to infect the brown widow spider, yet its consequences on the spider are unknown. In this chapter, I compare relative *Wolbachia* density in the abdomen, cephalothorax, and legs of brown widows, to determine if there is a difference in bacterial load between body regions. Information on bacterial distribution within the host allows us to make inferences on the relationship between the bacteria and the spider. No significant difference in *Wolbachia* density was found between the different body regions. The broad distribution of the bacteria may allow for host benefits like protection from parasitoids or fungal infections, which has been described in several species. There is also some indication that bacterial load varies among individuals, may explaining CI intensity variation among individuals reported in chapter 2.

INTRODUCTION

The brown widow spider, *Latrodectus geometricus*, is a species that has been introduced to every continent except Antarctica through human introduction and is thought to have originated in Southern Africa (Garb *et al.* 2004, Brown *et al.* 2008). In the Southeastern United States, the brown widow has become well established in the past 15 years after being limited to the southern Florida peninsula since the first sighting in 1935 (Brown *et al.* 2008, Vetter *et al.*

2012). Since 1990, the brown widow has expanded its range from southern Florida to Georgia, Alabama, South Carolina, Mississippi, Louisiana, Texas, and has also appeared in California and Hawaii (Pinter 1980, Garb *et al.* 2004, Brown *et al.* 2008). Brown widows were first seen in Georgia in the 1990's, and in 2007 it could be found up into north Georgia (Brown *et al.* 2008).

Wolbachia pipientis is a maternally inherited (Zug and Hammerstein 2015) alphaproteobacterium and in the order of *Rickettsiales* (Werren *et al.* 2008). *Wolbachia* was found to use the brown widow as a host and populations in the Southeastern United States vary significantly in infection frequency (Arrington 2014). *Wolbachia* is an intracellular parasite that can be transferred to offspring via the mother's egg cytoplasm (Shoemaker *et al.* 2000). Infected males are not capable of passing the bacteria to offspring due to sperm not donating cytoplasm during zygote formation (Werren 2008). *Wolbachia* can increase their fitness by acting as reproductive parasites within a host through four acquired mechanisms including male feminization, parthenogenesis, male killing, and cytoplasmic incompatibility (Werren 2008). Male feminization is when functional females develop from genetic males (Werren 1997, 2008). Parthenogenesis occurs when unfertilized eggs laid by a virgin haplodiploid female develop into functional progeny (Russell and Stouthamer 2011, Werren *et al.* 2008). Male killing is when there is a loss of male embryos laid by an infected mother (Sakamoto 2011, Werren 2008). Male killing and feminization have not been observed in *L. geometricus* (Arrington 2014). Parthenogenesis is also unlikely, as brown widows are not a haplodiploid species. Cytoplasmic incompatibility occurs when an infected male is mated with a female who is not infected or who contains a different *Wolbachia* strain, resulting in complete or partial embryonic death. Through these reproductive manipulations, *Wolbachia* can increase fitness by increasing the number of infected females in their clutch, allowing for the bacteria to spread through a population, with

strong CI leading to fixation within a population (Zug and Hammerstein 2015, Werren *et al.* 2008).

Wolbachia variants have been divided into eight supergroups A-H, with supergroups C and D found in filarial nematodes, while the other six supergroups, with A and B being the most common, found primarily in arthropods (Baldo *et al.* 2007, Werren *et al.* 2008). In 10 tested spider families, several were infected with either the A or B strain, while the other three belonged to supergroup G (Rowley *et al.* 2004). The *Wolbachia* that infects brown widows is classified into supergroup F, and sequencing indicated that the strain is unique to any currently known strains (J.S. Harrison unpublished data). Strains in supergroup F have been found in bed bugs (Hosakawa 2012), South African scorpions (Baldo *et al.* 2007), filarial nematodes (Casiraghi *et al.* 2005), bush crickets (Panaram and Marshall 2007), termites (Lo and Evans 2007), and lice (Covacin and Barker 2006). Even though this supergroup has been identified in several species, little is known about the host interactions of supergroup F strains. Hosakawa (2012) found that a supergroup F strain of *Wolbachia* endosymbiont aided in nutrient acquisition (vitamin B), promoting egg development in bed bugs. When antibiotics were used to eliminate the *Wolbachia,* eggs were inviable, which indicates an obligate mutualistic relationship between host and bacterium (Hosakawa 2012). In addition to reproductive and nutrient manipulations, *Wolbachia* has also been found to induce resistance to viruses, specifically Dengue, in *Aedes aegypti* mosquitoes, however this is not specific to supergroup F (Bian *et al.* 2010).

The relationship between brown widows and *Wolbachia* has not been fully described. However, partial CI has been found to occur (see chapter 2), indicating that there is some degree of reproductive manipulation associated with *Wolbachia* infection. Non-reproductive host interactions, including host nutritional enhancement like that seen in bed bugs, have yet to be

studied in the brown widow (Hosakawa 2012). Several studies have shown that reproductive manipulators are often concentrated in reproductive organs, whereas other studies have shown distribution throughout somatic tissue and reproductive tissue (Dobson *et al.* 1999, Veneti *et al.* 2003, Zouache *et al.* 2009, Hosokawa *et al.* 2010, Landmann *et al.* 2010, Casper-Lindley *et al.* 2011, Fischer *et al.* 2011, Andersen *et al.* 2012, Albertson *et al.* 2013, Strunov *et al.* 2013, Toomey *et al.* 2013, Roy *et al.* 2015). The aim of this study is to describe the distribution of *Wolbachia* among *L. geometricus* body regions, to determine if regions differ in bacterial load. Three different regions were selected for sampling: abdomen, cephalothorax, and legs. I hypothesize that there will be a significant higher bacterial load in the abdomen compared to the cephalothorax and the legs, as the abdomen contains the reproductive and digestive organs, while the legs will contain the least amount due to their lack of organs.

METHODS

Testing for Wolbachia

Female brown widows were collected from Statesboro, Georgia during 2016. A whole leg was removed from the specimen for DNA extraction and Polymerase Chain Reaction (PCR). The Genomic DNA™- Tissue Microprep kit (Zymo Research) was used for DNA extractions. To determine infected females, primers designed for the fructose-bisphosphate aldolase gene (FbpA) were chosen for a *Wolbachia* specific primer, as this primer has been found to amplify in all known strains of *Wolbachia* (Simões 2011, Vanthournout *et al.* 2001). The PCR products were run on a 1% agarose gel. Females were determined to be infected by *Wolbachia* based on the presence of PCR product for the FbpA gene. These females were allowed to produce egg sacs from breeding prior to being captured. The progeny from these different mothers were allowed to

grow and reach maturity. We selected five females from the progeny, with 4 being from the same mother and 1 being from a different mother as biological replicates.

Dissection, DNA Extraction, and Quantification of Extracted DNA

Wolbachia infected females were preserved in 70% ethanol. They were removed and dried by allowing the ethanol to evaporate for dissection. The spiders were placed in a sanitized petri dish and the legs were pulled from the cephalothorax at the trochanter, leaving the coxa attached to the cephalothorax. The abdomen and cephalothorax were separated using a scalpel that was sanitized via bleach and a Bunsen burner before each cut. The DNA extractions for each body segment were done by using the Zymo Research Genomic DNA™- Tissue Microprep kit. The extracted DNA was quantified using a Nanodrop (Thermo Fisher). The samples were then diluted to 20 ng/µl aliquots to standardize the amount of DNA present in the initial reaction.

Quantification of Wolbachia

Quantitative Polymerase Chain Reaction (qPCR) was performed using the QuantStudio 6 Flex Real-Time PCR machine (Life Technologies). *Wolbachia* specific qPCR primers were designed from hcpA gene sequences from *Wolbachia* found in the brown widow. hcpA is a conserved gene for an uncharacterized *Wolbachia* protein, and was used to quantify the amount of *Wolbachia* in specific body regions (Table 5). Spider specific primers were designed from NCBI GenBank sequences for the nuclear gene H3A (GenBank: FJ607605.1), a protein coding histone gene (Table 5). H3A was used as a reference gene control for DNA concentrations (Garb *et al.* 2004). These primers were tested for optimal annealing temperatures. SYBR-Green fluorescent dye (Qiagen), which binds double-stranded DNA molecules and fluoresces, was used for quantification. The recommended Qiagen protocol for a 20µl total volume was used. The

protocol includes 10 μL SYBR Green PCR Master Mix, 0.8 μL of 10 μM Forward and Reverse primers, 30 ng DNA template, and 7.5 μL RNase-free water for each reaction. The qPCR cycling program was adapted from Gay *et al.* (2015) including an initial denaturation step of 10 min at 95°C; 40 cycles of 1 min at 94°C, 1 min at 64°C, 1 min at 72°C, and a 20s-fluorescence reading step at 82°C; and a final elongation step at 72°C. ΔCT (hcpA-H3A) was determined by subtracting the respective H3A from hcpA to standardize the relative amount of *Wolbachia* with the reference gene. Each cell should have the same amount of the histone genes, so by standardizing the samples with H3A, we can see differences in quantification of *Wolbachia*. Three technical replicates were conducted for each spider body region. The ΔCTs were averaged to acquire a mean ΔCT for each individual spider's body region. The ΔCTs were analyzed with a one-way ANOVA.

RESULTS

There was variation among individuals, with individual 276.14 having a consistently higher ΔCT than the other individuals. This variation of 276.14 ΔCT compared to the other individuals is significantly higher for the abdomen and cephalothorax samples (Figure 6). An ANOVA was performed to determine if there was a significant difference in the pooled body region bacterial load between the two maternal lineages represented in the samples (274 n=4 and 276 n=1). A significant difference in maternal lineages was seen ($F= 33.8909$; $P<0.0001$*), however, more studies would need to be completed as the sample size for 276 was 1 (Figure 7). Sample 276 was removed as an outlier and the ΔCTs of the three different body regions from individuals of maternal line 274 were compared using an ANOVA and determined to not be significantly different, though the cephalothorax (average ΔCT= 5.352; standard deviation= 1.082) showed an average ΔCT that was higher than the abdomen (average ΔCT= 3.389;

standard deviation= 0.748) or the legs (average ΔCT= 2.825; standard deviation= 2.621) (Figure 8).

DISCUSSION

Despite the partial CI described in *L. geometricus* in Chapter 2 and the role of F-strain *Wolbachia* in nutrient acquisition in other species, *Wolbachia* is not at a higher density or limited to areas where reproductive or digestive tissues are located. The *Wolbachia* infecting brown widows are in the F-strain super group, this potentially novel mutualistic relationship could be like the mutualism that has been found in *Cimex lectularius*, a bedbug, with F-Strain *Wolbachia* (Arrington 2014, Hosokawa 2010). *Wolbachia* is essential for normal growth and reproduction in the bed bug through providing B vitamins (Hosokawa 2010).

PCR and fluorescent cytological approaches have allowed the distribution of *Wolbachia* within different body regions to be assayed and reveal that there is a broad distribution of the bacteria among somatic and germline tissues with region specific bacterial load varying among species (Pietri 2016). Some species of Tsetse fly have no infection in somatic tissue, but fruit flies, mosquitos, nematodes, bedbugs, ants, and termites, have been determined to have somatic tissue infection (Dobson *et al.* 1999, Veneti *et al.* 2003, Zouache *et al.* 2009, Hosokawa *et al.* 2010, Landmann *et al.* 2010, Casper-Lindley *et al.* 2011, Fischer *et al.* 2011, Andersen *et al.* 2012, Albertson *et al.* 2013, Strunov *et al.* 2013, Toomey *et al.* 2013, Roy *et al.* 2015). In bedbugs, *Wolbachia* densities in the bacteriome, a specialized organ that harbors bacterial endosymbionts, were found to be around 30 times higher than in the ovary and 2000-900000 times higher than in other organs (Hosokawa *et al.* 2010). If the bacteria were found to be more dense in a specific body region, we could draw potential conclusions as to the effect it has on its

host, whereas having a broad distribution does not allow us to eliminate or conclude potential host effects.

The broad distribution of *Wolbachia* within the brown widow indicates additional investigations of non-reproductive host interactions should be conducted. Protection from parasitoids (Oliver *et al.* 2003, 2005) and fungal infections through *Wolbachia* (Ferrari *et al.* 2001, 2004) has been described in several species. Baerg (1954) described a fungal outbreak that caused a severe loss of black widow spiders, *L. mactans,* while *L. geometricus* survived. This survival suggests immunity to the fungus. *Latrodectus geometricus* has been reported to displace the native *L. mactans* for unknown reasons. As they inhibit similar niches (Vincent 2008), anti-fungal properties could potentially be an advantage. *Wolbachia* has also been found to increase resistance to an arbovirus infection in a native *Wolbachia*-mosquito system (Glaser and Meola 2010).

Another non-reproductive host interaction that should be explored more is the impact of *Wolbachia* infection of temperature tolerance. In pea aphids found in South Florida, an endosymbiont provides benefits for heat tolerance, however, in novel northern environments, this benefit may become costly (Chen *et al* 1997, 2000, Russell and Moran 2006). In chapter 1, a trend if higher bacterial frequency with a decrease in latitude and a lower frequency with an increasing latitude is described. This suggests the possibility of a Wolbachia by environment interaction. This could be due to a difference in temperature tolerance, in which there may be an associated cost of having *Wolbachia* at lower winter temperatures, or a benefit of *Wolbachia* infection in the warmer southern populations.

An interesting outcome of this experiment was the significant differences in bacterial load between different maternal lineages (Figure 7). This difference in load could impact the

host/endosymbiont relationship through the severity of the reproductive manipulations that occur. *Wolbachia* density varies in wild-caught *Drosophila*, resulting in the efficiency of parasite transmission and intensity of male killing to fluctuate as well. (Unckless *et al.* 2009). This load difference may account for variation in levels of CI among crosses of ♀ x *w*♂ that occur in brown widows seen in Chapter 2. Variation in bacterial load among individual females could also result in generational dilution, a mechanism of symbiont loss, in some spider lineages (Shoemaker *et al.* 2000). Frequency of *Wolbachia* infection varies among Southeastern US *L. geometricus* populations (20% to 90%) (Arrington 2014). If the pattern of some maternal lines having a difference in the amount of *Wolbachia* is common, symbiont loss through generational dilution could cause the lack of association with mitochondrial haplotypes described in Chapter 1 and the distribution of *Wolbachia* frequency in *L. geometricus* populations. If there is a difference in individuals and populations with bacterial load, it would be expected for there to be an inconsistent amount of CI occurring and a loss of haplotype association, as some spiders may be getting a specific haplotype and very little bacteria and vice versa.

Another outcome of this experiment is the validation of DNA extraction and *Wolbachia* testing methods in the brown widow. As there was no significant difference between body segments both with and without an outlier, the method of DNA extraction from the legs of *L. geometricus* is confirmed as a suitable sampling method. This is important because spiders can be kept alive for breeding experiments when only a leg is removed for testing *Wolbachia* infection status.

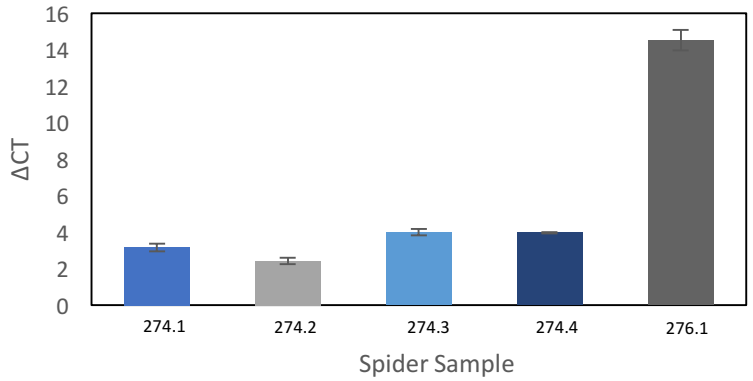
Wolbachia has been shown to induce partial CI in the brown widow spider. It will be important for future experiments on *L. geometricus*/*Wolbachia* interaction to use quantitative PCR (qPCR) to estimate the variation in relative load of *Wolbachia* among individual spiders

and offspring clutches. This is important to determine if different clutches are receiving the same amount of *Wolbachia* or if there is a clutch or individual effect on the number of bacteria being passed to offspring. Future directions would include further dissection of spiders to isolate various organs and determine via qPCR if there is a difference in *Wolbachia* load. This would allow us to draw further conclusions about the potential mutualistic relationship between the bacteria and *L. geometricus*.

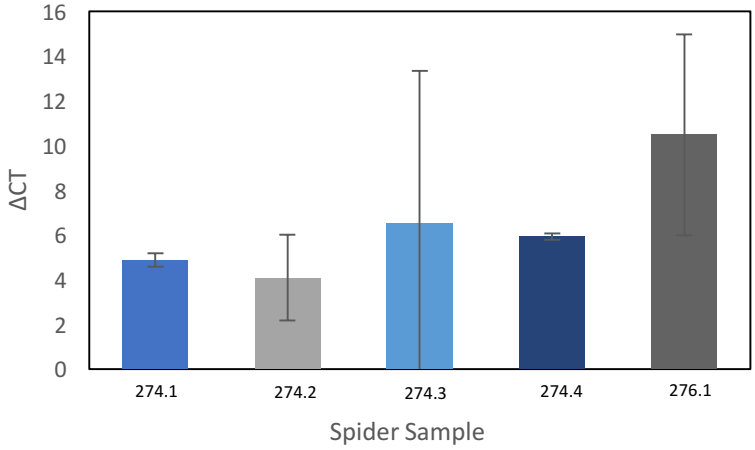
Table 5. qPCR Primers for *Wolbachia* Quantification

Gene	**Forward**	**Reverse**
H3A Histone	CACCAAAGCTGCACGTAAAAG	AGGGAAGTTTGCGGATGAG
hcpA *Wolbachia*	CAAATAACCGCAACCGAACTG	GTGCCCTCTGCTTTATAGACG

(A)



(B)



(C)

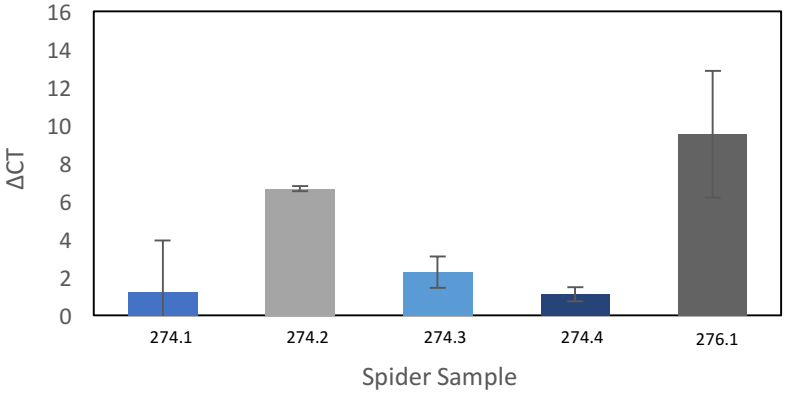


Figure 6. Average ΔCTs of the technical replicates for each individual for different body regions to quantify *Wolbachia*. (A) Average abdomen ΔCTs for spider samples ± s.e.

The 276.1 sample has a significantly larger ΔCT than the other samples (P = 0.0009*). (B) Average cephalothorax ΔCTs for spider samples ± Standard Error. The 276.1 sample has a significantly larger ΔCT than the other samples (P = 0.0241*). (C) Average leg ΔCTs for spider samples ± Standard Error. There is no difference between the spider samples (P = 0.1062).

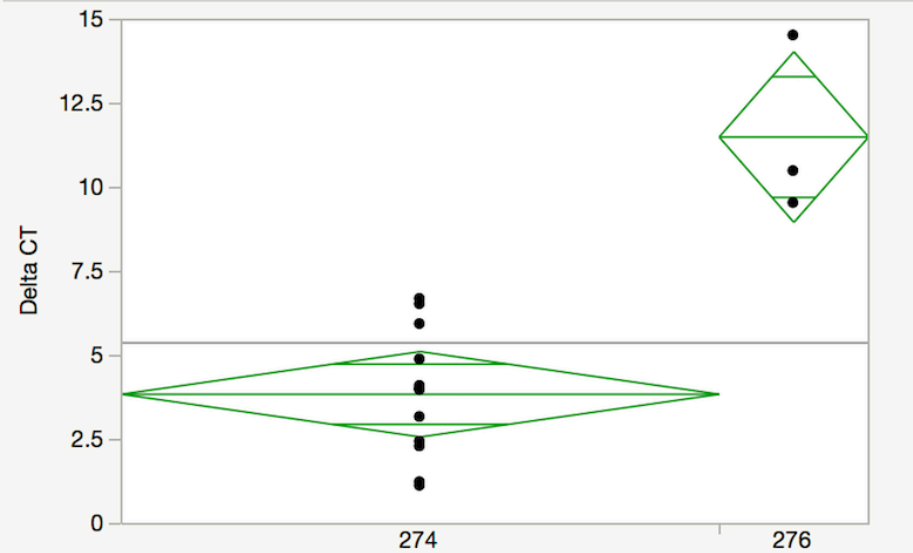


Figure 7. Average ΔCTs of the two different maternal lineages. There is a significant difference in bacterial load between maternal lineages (F= 33.89; P<0.0001*).

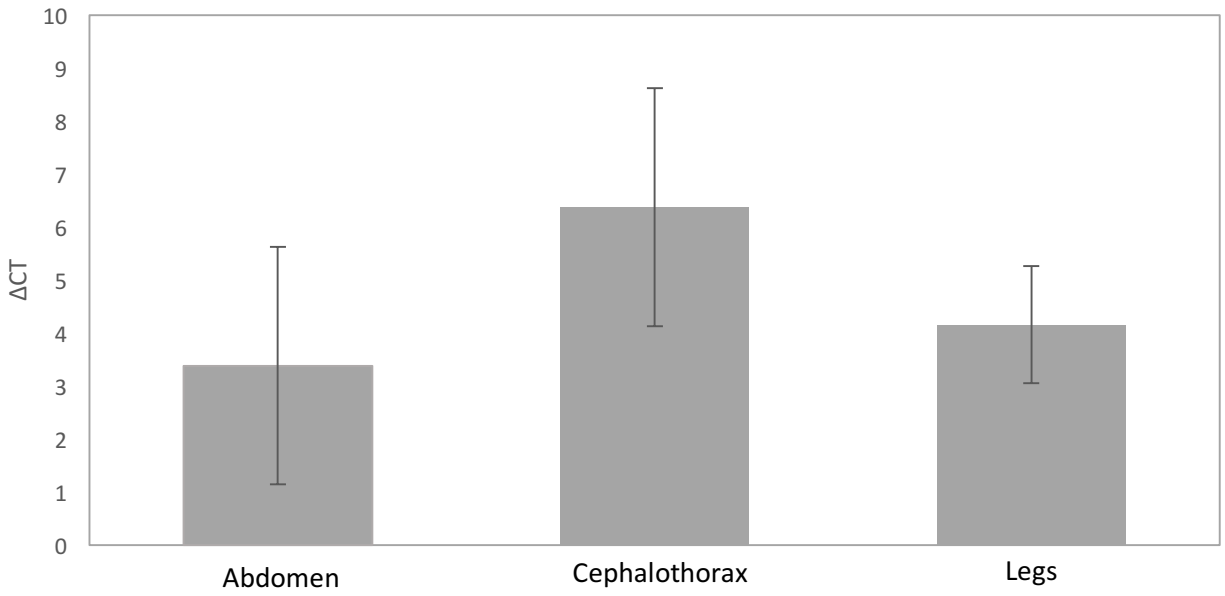


Figure 8. Average ΔCTs of three different body regions with 276 samples removed. There is no significant difference in bacterial load between different body regions (F= 2.45; P= 0.14).

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DISCUSSION

Examining the potential for linkage between *Wolbachia* and mitochondrial haplotypes in the brown widow spider (Chapter 1) gives us insight into the interactions between host and symbiont, as well the evolutionary history of the relationship. Additionally, investigating the potential for cytoplasmic incompatibility to occur (Chapter 2) provides insight into the effects of reproductive manipulations *Wolbachia* has on brown widow life history and population dynamics. Finally, determining where *Wolbachia* is localized within the body of the spider (Chapter 3) delivers insight into the potential for fitness effects that are associated with infection. Combined, this research gives overall insight into the relationship between the brown widow and *Wolbachia*, furthering the knowledge of bacterial endosymbionts.

In Chapter 1, there was no linkage seen between *Wolbachia* and mitochondrial haplotypes in the brown widow. When looking at *Wolbachia* frequency over time, there was no consistent pattern, as an increasing trend in frequency was seen in Louisiana, while Georgia showed a decrease. There was also no correlation between specific haplotypes or levels of haplotype variation with infection status of spiders. This lack of linkage could be explained by the infection being old resulting in a loss of selection for the infection and a weakening of the associated reproductive manipulations. It could also be explained by horizontal transfer, which has been shown in different systems, but is rare.

In Chapter 2, partial CI was observed, as complete embryotic death was not observed in a cross between an infected male and uninfected female. This could be attributed to different bacterial densities, as a certain threshold of density may be required to modify the paternal genetic material. Temperature has also been found to influence CI levels. With reduced virulence associated with partial CI, a decrease in selective pressures on the bacteria resulting in

a loss of fixation can occur. Low intensity of CI is likely a strong factor in the lack of linkage between *Wolbachia* and mitochondrial haplotype diversity, as well as the lack of patterns in the haplotype gene tree in Chapter 1.

In Chapter 3, no significant difference in *Wolbachia* density was found between regions, however, there was slightly higher relative density in the cephalothorax. More experiments would need to be conducted to determine if that increase is correlated to a host interaction caused by the bacteria. The broad distribution of the bacteria among body regions may allow for host benefits like protection from parasitoids or fungal infections. One interesting outcome of the experiment was the difference in bacterial loads based on maternal line. This could account for the variation in CI levels seen in Chapter 2. Bacterial load variation could also result in generational dilution and a mechanism of symbiont loss in some lineages. This is consistent with the lack of correlation to mtDNA variation seen in the gene tree in Chapter 1 that could be explained by a loss of *Wolbachia* infection from the population. This experiment also validates our DNA extraction method to test for *Wolbachia* infection. Legs are taken for extraction and detection purposes so that the spider can live and be used in other experiments.

These experiments demonstrate that there is no linkage between *Wolbachia* and mitochondrial haplotypes in the brown widow, which could be due to a loss of infection due to decreasing selective pressures. Weak cytoplasmic incompatibility was seen, which could be due to temperature changes and cause a decrease of selective pressures resulting in a loss of fixation. There was also no difference in *Wolbachia* density in different body segments. No localization to the reproductive organs can aid in explaining weak CI, with a broad distributing potentially implying parasitoid and fungal protection. Differences in maternal load could also account for the variation in CI, as well as the loss of selective pressures resulting in a lack of linkage.