ABSTRACT

FISHER, MICHAEL LAURIN. Studies on the Symbiosis of *Wolbachia* and *Cimex lectularius*: Growth Kinetics, Horizontal Transmission, and Influence on Virus Replication. (Under the direction of David W. Watson and Coby Schal).

Over the past two decades, the world has experienced a profound resurgence in bed bug (*Cimex lectularius* L.) infestations. This resurgence and persistent bed bug infestations has generated an unparalleled need for research in bed bug biology. The main objectives of this dissertation were to expand current knowledge of the symbiotic association between the bacteria *Wolbachia* and *Cimex lectularius* that investigated the following: quantification of *Wolbachia* and growth kinetics over the course of bed bug development cycle, evaluate whether *Wolbachia* can be horizontally-transmitted via sexual or environmental means, and whether *Wolbachia* can suppress ingested viruses as observed in other blood-feeding insects. We examined the amounts of *Wolbachia* in immature and adult bed bugs at various intermolt and blood-fed stages. While the growth kinetics of symbiotic microorganisms has been studied in other insect systems, it has been neglected in bed bugs. Recent literature has reported variation in both infection frequency and the relative abundance of *Wolbachia* in field-collected samples of bed bugs. We extracted DNA from the Harold Harlan and Jersey City strains of bed bugs and obtained absolute quantification of the 16S copy number of *Wolbachia* in 1^{st} instars, 5^{th} instars, and adults using a Droplet Digital PCR (ddPCR) system optimized for *Wolbachia* detection. Our results highlight that *Wolbachia* is dynamic during bed bug development, changing substantially relative to life stage, intermolt stage, and blood-fed status. This work is described in Chapter 2.

We also investigated the possibility of horizontal transmission of *Wolbachia* between mating adults and to the progeny of *Wolbachia*-free females that mated with normal males. Mutualistic associations of *Wolbachia* within insects are maintained throughout natural populations almost exclusively through vertical transmission from mother to offspring, certainly in the obligate mutualism system in *Cimex lectularius*. In some blood-feeding insects, horizontal transmission of facultative *Wolbachia* occurs mainly via sexual transfer from an infected male to uninfected female. Horizontal transmission of *Wolbachia* in bed bugs is poorly understood. We used an established line of *Wolbachia*-free bed bugs to set up assays to examine transfer of *Wolbachia* from female to male, male to female, and female to male to female. Quantification of *Wolbachia* was performed with Droplet Digital PCR that was highly sensitive to both bacterial and bed bug DNA even at low concentrations. Our results suggest that *Wolbachia* could not re-establish in *Wolbachia*-free adults after copulation with *Wolbachia*-infected mates, but *Wolbachia*-infected males appeared capable of transferring the endosymbiont to their progeny, either through the fertilized eggs of the *Wolbachia*-free mothers or through subsequent contact of the nymphs with the male or his feces. This work is described in Chapter 3.

Lastly, we evaluated the influence of *Wolbachia* on virus replication within *Cimex lectularius*. Despite more than 100 years of inquiry into bed bugs as disease vectors, they still have not been conclusively linked to any disease or pathogen. *Wolbachia* induces resistance to viruses such as Dengue, Chikungunya, West Nile, and Zika, and primes the insect immune system in other blood-feeding insects. We fed an established line of *Wolbachia*-cured bed bugs and a *Wolbachia*-positive line a virus-laden blood meal that used feline calicivirus as the model, and quantified the amount of virus over five time intervals post-feeding. There

was a significant effect of time as the amount of virus declined by ~90% over 10 days in both groups, but no significant difference in virus titer was observed between the *Wolbachia*-positive and *Wolbachia*-free groups. This work is described in Chapter 4.

Studies on the Symbiosis of *Wolbachia* and *Cimex lectularius*: Growth Kinetics, Horizontal Transmission, and Influence on Virus Replication.

by
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APPROVED BY:

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DEDICATION

To my parents Mary and Bradley, my wife Nosika, and my two adorable children Natalie and Alexander for their unconditional love and support.

BIOGRAPHY

Michael Laurin Fisher was born on September 13, 1979 in Marshalltown, Iowa, and grew up on a farm with his parents and younger sister Anna in the nearby town of Montour, Iowa. His family has farmed in central Iowa for more than 100 years, and two century farms are still owned by the Fishers there. He helped his dad, grandfather, and uncle raise cattle, pigs, as well as corn and soybeans, but also sweet corn and melons, and bailed hay most summers. He attended the Montour Elementary School through 5th grade where he was first introduced to the world of insects from his 4th and 5th grade teacher, Mr. Jim Little, and began what was to become a life-long love of arthropods. He attended middle and high school at South Tama, in Tama-Toledo, Iowa, where he continued to pursue his interest in insects and science, being an avid collector of mostly Lepidoptera (butterflies and moths), but also Coleoptera (beetles).

After graduating high school in the top 6% of his class and with 13 college credits (College Algebra, Pre-Calculus, and Calculus), he attended Iowa State University in the fall of 1997 working toward a Bachelor of Science in Animal Ecology-Pre-veterinary concentration. While his love of animals and drive to become an exotic animal veterinarian was strong, he ultimately found his passion for studying insects of medical and veterinary importance was far greater, and continued work on a dual Bachelor's degree in Entomology. He graduated in 2003 with distinction. After graduation, he worked briefly for the USDA-APHIS, and for United Parcel Service in Des Moines, Iowa on weeknights part-time.

Although both jobs provided ample income for a newly-graduated college student, Michael was anxious to pursue work in an entomology-related capacity. He learned that both

the U.S. Navy and U.S. Air Force had Active Duty entomologists, and began researching if this would be a good career move. The Navy is the only branch that contacted him and was eagerly recruiting him upon learning of his BS in Entomology from Iowa State. However, an Active Duty entomologist in the Navy required a Master's degree. Michael applied for and was accepted into the Navy's Health Service Collegiate Program where he would enlist on Active Duty as an Officer Candidate with full pay and benefits while pursuing a Master's of Science in Entomology at the school of his choice. He was accepted at the University of Nebraska-Lincoln in the summer of 2005, and began working towards an MS degree in the fall of that year under the direction of Drs. Leon Higley and John Foster focusing on forensic entomology. During his two years at Nebraska, Michael was active in the Entomological Society of America, and competed in student presentation competitions, as well as the Linnaean Games entomology quiz-bowl regional and national competitions. He helped the University of Nebraska win their first National Linnaean Games championship title in San Diego, California in 2006, as the only MS student on the team, being the 'expert' in medical-veterinary insects. In 2007, he passed the general and specialist exams to earn board certification and has maintained his status since. He graduated and was commissioned as Lieutenant JG in the U.S. Navy Medical Service Corps in December of 2007.

He attended Officer Development School from January to February 2008, followed by his first tour as Entomology Division Officer at the Navy Environmental and Preventive Medicine Unit 2 in Norfolk, Virginia. While there, he provided entomology technical assistance to the Navy's 2nd Fleet and deployed two times, once in 2009, aboard the USNS COMFORT (T-AH 20) in support of strategic partnership building to seven Central and

South American countries, and again in 2010 as Team Leader, Entomology Block with the Forward Deployable Preventive Medicine Unit in support of Operation Iraqi Freedom and Operation Enduring Freedom to Kuwait and Afghanistan. His second tour of duty, from 2011-2014, was with 2d Medical Battalion in Camp Lejeune, North Carolina. There he served as entomologist for Marine Corps Installations East, Officer in Charge of the Preventive Medicine Unit, Company Commander of Alpha Surgical Company, and Operations Officer for the battalion. He deployed there as well in 2013 as Officer in Charge of the Preventive Medicine Detachment at Camp Leatherneck, Afghanistan, in support of Operation Enduring Freedom and International Security Assistance Force.

During his 2013 deployment, he applied for and was later accepted into Navy Medicine's FY14 Duty under Instruction program to pursue a 3 year Ph.D. in Entomology funded by Navy Medicine at the school of his choice while on Active Duty. He simultaneously applied at North Carolina State University and was accepted in the fall of 2013. In July of 2014, he began coursework towards his doctorate, and developed projects that studied various aspects of microbial symbiosis in bed bugs. In September of 2016, he was promoted to his current rank of Lieutenant Commander, and after graduation in 2017, will report back to the fleet as Director of the Vector Biology Department at the Navy Medical Research Unit 6 in Lima, Peru. His work on insects of medical and veterinary importance will continue, as he will be studying mosquitoes, sand flies, filth flies, and other significant pests, as well as evaluating novel chemistries as repellents and insecticides. Michael has been truly honored for the opportunity to serve his country, and his educational experiences at North Carolina State University have been nothing short of exceptional.

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I would like to thank several individuals, who without their expertise and guidance, the completion of this work and dissertation would not have been possible. I am extremely grateful to all my committee members – Wes Watson, Coby Schal, Jay Levine, Marcé Lorenzen, and Logu Ponnusamy – for their valuable time, unwavering devotion, guidance, and expertise that has helped shape this innovative work. I especially thank Wes Watson for his outstanding mentorship, generosity, honesty, and sense of humor, as well as his interpersonal skills, availability throughout my graduate career, assistance with project development, and ability to recognize talent from non-traditional educational backgrounds. I wish to extend a huge thanks and sincerely appreciate all of the dedication from my co-advisor Coby Schal, for his time, effort, and initiative spent on project development, experimental design reviews, and critiques of my scientific writing. Wes and Coby provided significant contributions and outside perspective on many aspects of my research and scientific career. I thank Jay Levine for serving as my Graduate representative, and for his help getting the virus work started off in the right direction in collaboration with the Clinical Virology lab in the College of Veterinary Medicine at NCSU. I thank Marcé Lorenzen for her assistance with various project aspects and lending her expertise in insect molecular biology techniques. I sincerely thank Logu Ponnusamy for his inspiration that sparked my interest in insect microbial symbiosis, his outstanding mentorship in insect microbiology and molecular techniques, as well as his generosity and devotion toward my research. To my entire committee, thank you from the bottom of my heart.

I thank Jim Guy from the Clinical Virology lab at NCSU's College of Veterinary Medicine for the use of his lab and equipment, and for providing his expertise and assistance with the virus work. I also thank the Clinical Virology lab technicians Melissa West and Leah Scarborough for all of their help and assistance with the virus project. I thank Matthew Breen from the Department of Molecular and Biomedical Sciences at NCSU's College of Veterinary Medicine for the use of his lab and equipment for various aspects of my research. I thank Hiroyuki Mochizuki for his expertise and assistance with the ddPCR.

I thank the entire Entomology and Plant Pathology staff, especially Gene Dupree, Rick Santangelo, and Steve Denning. The help of Rick Santangelo, whose technical expertise, support with colony-rearing, and willingness to help whenever needed, proved invaluable throughout my graduate career. I extend a huge thanks to Steve Denning for his countless hours of assistance with numerous aspects of my research. I also thank the Blanton J. Whitmire endowment, the US Department of Housing and Urban Development Healthy Homes program (NCHHU0017-13), the Alfred P. Sloan Foundation (2013-5-35 MBE), the NCSU W. M. Keck Center for Behavioral Biology, the Pi Chi Omega National Pest Control Fraternity, the NCSU Department of Entomology and Plant Pathology for funding my research. I also thank the U.S. Navy Medicine Professional Development Center for providing 100% tuition funding and a generous monthly stipend.

I am eternally grateful for the love and support of my family. I thank my mother and father immensely for instilling such a resilient work ethic in me. And I cannot express my gratitude enough to my beautiful and amazing wife Nosika, for her unconditional love and support throughout this endeavor.

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CHAPTER 1.

Microbial Symbioses of Hemiptera: Historical Perspectives, Recent Focus, and Future Directions

Introduction

Insect microbial diversity, endosymbiotic relationships, and how microorganisms interact and influence their macroorganism hosts has garnered a significant amount of attention in recent years. In a healthy insect, the microbiota can comprise up to 10% of the insect's biomass, and in several examples, bacteria have evolved mutualistic and commensal relationships with their insect hosts to the level of interdependence amongst the species. It is estimated that endosymbiotic relationships with microorganisms exist in perhaps as much as 15% of all insect species (Douglas 1998). The continual advancements in molecular techniques coupled with high throughput sequencing technology has enabled scientists to delve further and further than ever before into this fascinating field of insect-microbial interactions. This purpose of this review is to provide an overview that summarizes the historical and recent literature of Hemipteran insects and the relationships with their resident microorganisms.

Historical Perspectives

1850-1950

Identification and histology of resident microorganisms

One of the oldest, most-widely studied group of insect-microbe interactions exists in the Hemiptera-Homoptera complex. In the early part of the 20th Century, the German zoologist and cell biologist Paul Buchner (1886-1978), referred to by many as the father of systematic symbiosis research, extensively studied and published on the bacterial and fungal relationships of arthropods (Gibson and Hunter 2010, Sapp 2002, Buchner 1965). *Buchnera*

aphidicola, the primary endosymbiotic bacteria of the pea aphid *Acyrthosiphon pisum*, was named after him by Paul Baumann (Sapp 2002). It was this work on aphid-*Buchnera* hereditary symbiosis by Buchner that led to the broad expansion of insect-microbial symbiosis research, and solidified the importance of symbiosis in evolution (Sapp 2002).

Yet even before the legacy of Buchner, a handful of scientists were exploring microorganisms associated with the Hemiptera, and the intracellular 'organisms' of aphids were among the first to be studied (Steinhaus 1940). These early 19th Century researchers included Dufour in 1833 who characterized 'sac-like' appendages on the posterior midgut of certain Hemiptera (Heteroptera), and Leydig in 1850 who observed certain organs in aphids that were subsequently termed 'symbiotic organs,' 'green body,' and 'mycetome' by several researchers that followed him (Steinhaus 1940, Steinhaus et al. 1956). The distinctively colored green mass of symbionts of the developing parthenogenetic aphid egg was studied in more detail in 1858 by Thomas Henry Huxley (1825-1895), effectively known as "Darwin's Bulldog" for his feverish advocacy of Charles Darwin's theory of evolution, who termed these yolk-like symbiotes 'pseudovitellus' (Lanham 1968). Following Huxley, in 1866, Metschnikov called it 'secondary yolk,' and both he and Witlaczil in 1884 were convinced it arose from direct invasion and modification of follicle cells (Lanham 1968). Five years later in 1889, while studying the embryology of cockroaches, American entomologist William Morton Wheeler (1865-1937) termed these bacteria-like particles in the egg cytoplasm 'Blochmann bodies' after their discoverer, German zoologist Friedrich Blochmann (1858-1931) in 1887, but this term was later abandoned due to the lack of cultivation in media (Lanham 1968).

Stephen Alfred Forbes (1884-1930), recognized by the National Academy of Sciences as the founder of the science of ecology in the United States, and economic entomologist, published two works before the turn of the Century on microbes associated with Hemipterans; one on the 'bacterium parasite' of the chinch bug (Forbes 1882), and 'bacteria' in digestive organs in members of the families Scutelleridae, Pentatomidae, Lygaeidae, and Coreidae (Forbes 1892, Steinhaus 1940). Umberto Pierantoni (1876-1958) discovered symbiotic organs in the scale insect *Icerya* in 1909 (Sapp 2002). In 1910, Pierantoni and Moravian embryologist Karel Šulc (1872-1952) interpreted the 'pseudovitellus' of Homopterans as a primitive gland organ in the gut colonized with intracellular symbiotic microbes, and deemed them 'yeast fungi' or mycetomes (Zchori-Fein and Bourtzis 2012, Sapp 2002, Buchner 1965). In fact, the term mycetome was used throughout the 20th Century to describe the symbiotic organ of many species of cockroaches, leafhoppers, and aphids (Sapp 2002). The mycetome was referred to as an assemblage of the Blochmann particles or somatic cell 'mycetocytes' (Lanham 1968, Musgrave 1964). Glasgow (1914) described in great detail the bacteria-containing ceca of Heteroptera (Hemiptera), and Kuskop (1924) followed up on Glasgow's work. Free-living, aerobic microorganisms that fix nitrogen in the genus *Azotobacter* were discovered in aphids in 1914 by Peklo, but this work was not published until 1953 (Lanham 1968, Peklo 1953). Uichanco (1924) studied aphid embryology and demonstrated the relationship between the secondary yolk and the mycetome of the adult aphid.

Credited with the term "hereditary symbiosis," Buchner confirmed that these inaccurately described organelles were actually bacteria adapted to intracellular life (Sapp 2002, Buchner

1965). In 1920, he studied the symbiotic relationships of blood-sucking insects, including bed bugs in Hemiptera, and assumed the bacteria played a role in the digestion of red blood cells (Sapp 2002). At about the same time, others were also studying "rickettsia-like" microorganisms of Hemipterans (Hertig & Wolbach 1924, Arkwright et al. 1921). These intracellular microorganisms isolated from the mycetome and ovaries of the bed bug *Cimex lectularius* (L.) were classified as *Rickettsia lectularia* (Steinhaus 1941, Pfeiffer 1931, Hertig & Wolbach 1924). By 1921, Buchner had published what he considered symbiosis in aleyrodids, aphids, cicadellids, coccids, cicadas, and psyllids (Steinhaus 1940). By 1925, he had investigated and described the inheritance of multiple symbiosis in singing cicadas, and even examined the polysymbiotic relationship in them where three different microbes were present in egg cells simultaneously (Sapp 2002). Although Buchner's definition of symbiosis fit well in the Homoptera, some argued that symbiotic terminologies and definitions can be subjective and confusing (Houk & Griffiths 1980, Starr 1975). Buchner's profound research even led to the concept of host control of their symbiotes by limiting their reproduction. His extensive observations on insect symbiosis and mycetocytes concluded that binary fission occurred at a relatively low rate compared to free-living bacteria (Houk and Griffiths 1980).

Beginning in the 1940s, the world-renowned American geneticist, molecular biologist, and student of Buchner, Hermann Joseph Müller (1890-1967), studied extensively what Buchner referred to as the 'fairyland of symbiosis' in the Hemipteran suborder Auchenorrhyncha (cicadas, spittlebugs, and hoppers), where he concluded that the majority of species contained multiple symbiont types (Zchori-Fein and Bourtzis 2012, Moran et al. 2005, Müller 1962). Müller examined 405 species of Hemipterans and also discovered that

348 of those had two or three distinct symbionts based on morphological characteristics (Moran et al. 2005). The morphological studies by Müller in 1940 and Resühr in 1938 facilitated the recognition of these symbionts as bacteria (Zchori-Fein and Bourtzis 2012).

Edward Steinhaus (1914-1969), a well-known American insect pathologist, worked for several years in the U.S. Public Health Service studying rickettsial diseases and the relationships between intracellular microorganisms and ticks (Sapp 2002). In 1939, as a post-doctorate, Steinhaus received the Muellhaupt Fellowship for his work on insect microbiology. He examined the bacteria associated with 30 species of insects collected near the Ohio State University or from the Entomology department's specimens, and cultured and categorized 83 bacterial species from digestive tracts, ovaries, and Malpighian tubules from seven families of Hemiptera and Homoptera: Reduviidae, Lygaeidae, Miridae, Pentatomidae, Cimicidae, Aphididae, and Cicadidae (Steinhaus 1941). The majority of work done by Steinhaus was devoted to an increased understanding of the biological relationships between insects and microorganisms, as well as the connection between the symbiotes of mealybugs with their phylogenetic and systematic character traits (Sapp 2002). During World War II (1939-1945), Steinhaus was able to write a major book titled *Insect Microbiology*, which included chapters on 'bacterium-like' symbiotes and another chapter on 'yeast-like' symbiotes (Sapp 2002). The 1946 book highlighted Buchner's early contributions to insect-microbial interactions, fostered a shared dialogue, and solidified a professional relationship between the two scientists. Steinhaus also isolated bacterial symbiotes from the caeca of a stinkbug, squash bug, cactus bug, and plant bug and identified them as *Pseudomonas* (Steinhaus et al. 1956). As research on microbial symbiosis within Hemiptera continued in

the latter half of the 20th Century, the focus gravitated towards the characterization of the ultrastructure of these microorganisms, their evolutionary origin, and what their purpose or defined role was.

1950-1980

Early Controversy

The early literature (pre-1965) is wrought with controversy regarding the microbial origin of symbiotes, specific mode of transmission, and whether these microorganisms were extracellular or intracellular. This is not unexpected, since the majority of Buchner and fellow researcher's work was carried out before the renowned 1953 discovery by Francis Crick and James Watson on the structure of deoxyribonucleic acid (DNA). Researchers' failure to accurately detect symbiote DNA in several Homopteran species contributed to the microbial origin controversy of these microorganisms (Houk and Griffiths 1980). Additionally, all of the research and experiments were conducted using light microscopy, and while novel in that era, became eclipsed by electron microscopy as technology progressed. Koch (1967) cited many reports on the isolation and in vitro cultivation of several species of bacteria in Homoptera that were carried out prior to 1955. However, studies that followed after 1955 were done with improved molecular techniques, and some tended to question the validity and methodology of these early reports (Brooks & Richards 1966). On the aspect of mode of transmission, Buchner was convinced that transovarial transmission occurred in the aphid-symbiont association and in other Homopterans, and symbionts left the mycetocytes that surrounded the ovarioles and migrated between the follicle cells to enter developing

oocytes (Lanham 1968, Gier 1936). Work by Stevens in 1905 on the parthenogenetic eggs of *Aphis rosae* concluded that the symbiont mass was transferred through an opening in the ovariole from the maternal mycetocyte, which he termed a 'vitellarium' (Lanham 1968). Buchner later illustrated this biological phenomenon of symbiont transfer into the parthenogenetic eggs in mycetocytes of *Aphis sanborni* (Buchner 1921). Initial studies of the mycetomes and other supposedly analogous microorganisms in other insects showed them to reside intracellularly in some insects, but extracellularly in others, which naturally invited confusion (Blewett & Fraenkel 1944, Brooks 1956, Buchner 1965, 1921, Bush & Chapman 1961, Gier 1936, Glaser 1920, Gresson and Threadgold 1960, Koch 1960, Lanham 1968, 1952, Musgrave and Miller 1956, Trager & Lanham 1952). However, Buchner's definitive and comprehensive work ultimately illustrated the intracellular characteristic of these microorganisms.

Ultrastructure, function, and physiology of endosymbionts

Literature from the late 1920s through 1960 is sparse on the subject of endosymbiotic relationships relative to the morphology and specific function of endosymbionts within Hemiptera. The number of publications examined for this review from 1921-1960 average 7.25 per decade (**Figure 1.1**). During this period, little overlap existed between the fields of microbiology and entomology. However when the two fields did overlap, microbiologists and entomologists avoided this shared area, possibly due in part to unfamiliarity with each other's respective field (Steinhaus 1960). World War II occurred during this period, and it is reasonable to speculate that the sparse number of publications in the realm of microbial

symbiosis in insects during this era is linked to the funds for such research being devoted toward the war effort.

Yet despite a major world conflict and limited funding sources, research on examining the function of symbiotic microorganisms in Hemiptera progressed, particularly on evaluating aposymbiotic insects and further exploration of the nutritional role of symbionts. Following Buchner's early work, a symbiotic bacterial organism that occurs constantly in the blood-sucking insect *Rhodnius prolixus* was thought to contain vitamin B as a dietary requirement, and thus digested in the intestine following a bloodmeal (Wigglesworth 1936). A few years later, Wigglesworth followed up on his previous work with *R. prolixus*, and among the discoveries was that vertical transmission of the symbiont *Actinomyces rhodnii* did not occur, speculating that it was obtained via coprophagy (Brecher and Wigglesworth 1944). Aposymbiotic *R. prolixus* nymphs grew and molted normally to 4^{th} and 5^{th} instars, but very few became adults without *Actinomyces*. Normal growth and egg production resumed when recovery occurred with the symbiont. More and more studies continued to support the view that symbionts function in the synthesis of essential vitamins, such as B complex in hematophagous Hemipterans, amino acid synthesis in sap-feeding Hemipterans, and lipid synthesis in aphids, and had hypothesized roles as a source of energy and even osmoregulation (Baines 1956, De Meillon and Goldberg 1947, Henry 1962, Houk and Griffiths 1980, Trager 1947). Smith (1948) was unable to demonstrate the atmospheric nitrogen fixation by symbiotic microorganisms of aphids using the stable isotope ^{15}N, despite early theories on the function of aphid symbionts. In the late 1950s, Insect Pathology became its own, distinct area, as research intensified on the pathogenicity of microorganisms in

insects (Steinhaus 1960). The fitness, fecundity, behavior, and overall consequences of several arthropod groups, including Hemipterans, treated with antibiotics, were thoroughly evaluated, and the research offered significant support for the nutritional role of symbionts (Baines 1956, Brooks 1963, Houk and Griffiths 1980, Lake and Friend 1968,).

Interest in microbial interactions eventually gained considerable momentum by mid-Century, arguably as a result of Buchner's most noteworthy publication in 1965, and the significant contributions of Müller in the fields of genetics and general biology. Beginning in the 1960s and continuing over the next two decades, an era of significant study was devoted to an increased understanding of the ultrastructure, development, and physiological/biochemical roles of endosymbionts in insects. As outlined in the review of intracellular symbionts associated with Homoptera by Houk and Griffiths (1980), the literature from 1965-1980 is clearly dominated by two distinct groups of economically-important Hemiptera, aphids and leafhoppers. However, a small number of publications did evaluate symbiotic associations in hematophagous Hemipterans (Harrington 1960, Cavanagh and Marsden 1969, Chang and Musgrave 1973, Chang 1974, Hill et al. 1976). Chang and Musgrave (1973) followed up on earlier work in bed bugs by Steinhaus (1941), and studied in greater detail the morphology and ultrastructure of the mycetome of *C. lectularius*. Like Steinhaus (1941), who repeatedly isolated the bacteria *Corynebacterium paurometabolum* that was presumed not to be the 'symbiote' of bed bugs, they were able to identify two morphologically-distinct types of 'rickettsia-like' microorganisms; one that was small and rod-shaped, and the other pleomorphic and globular (Chang and Musgrave 1973). In eight species of reduviids, the bacterial flora diversity was examined and found to be limited to one

or two species per insect, but the bacteria *Enterococcus faecalis* was isolated from both *R. prolixus* and *Triatoma infestans* (Cavanagh and Marsden 1969).

By the late 1960s, the general use terms mycetomes, symbiotes and 'plasmids' were commonly used in reference to arthropod structures that house microorganisms (Chang and Musgrave 1973, Lederberg 1952, Steinhaus 1967). Yet as more knowledge was obtained on the ultrastructure of these microorganisms, researchers began to refer to these specialized host cells as 'bacteriocytes,' harbored in aggregations as 'bacteriomes' (Buchner 1965, Chang and Musgrave 1969). Bacteriomes and *Buchnera* are nearly universal within the true aphids (Aphidoidea), but exist as extracellular yeast-like symbionts in the body cavity other aphid groups (Cerataphidini) likely as a result of a loss from a *Buchnera*-containing ancestor (Baumann et al. 1995, De Clerck et al. 2014). Griffiths and Beck (1973) confirmed with SEM what was not possible with light microscopy the presence of multiple morphotypes in aphids. The comprehensive review of literature from 1965 up to 1980 by Houk and Griffiths (1980) discusses in great detail the ultrastructure, morphology, physiology, and function of Homopteran symbionts.

1981-1990

For an unknown reason, literature on Hemipteran symbiosis during this decade dips slightly. Regardless, several notable and extensively-cited works were published during this period on mycetocyte or bacteriocyte-associated organisms and their insect host (Dasch et al. 1984, Douglas 1989, Douglas and Smith 1989, Smith and Douglas 1987, Unterman et al. 1989). Angela Douglas and Hajime Ishikawa, now two world-renowned researchers in the field of

insect-microbial interactions, became key figures during the 1980s on this subject. Their combined works from 1981-1990 totaled half of all literature sources examined for this review. Once again, the primary organisms of study for symbiotic relationships during this period were aphids and hoppers, but other groups of Hemipterans started to gain more attention as the focus shifted towards in-depth examinations of the biochemical aspect of the host-symbiont relationship. A few studies expanded research away from the aphid and hopper groups and characterized intracellular microorganisms of Pyrrhocorids and Psyllids (Chattopadhyay 1984, Chattopadhyay and Choudhuri 1981, Haas and Konig 1987, Waku and Endo 1987). Additionally, it was discovered that growth and development of reduviid nymphs was severely diminished when individuals were fed on rabbits immunized against the *R. prolixus* bacterial symbiont *Nocardia rhodnii* (Ben-Yakir 1987).

Host nutrition and amino acid synthesis via endosymbionts

Several studies addressed what specific amino acids or proteins endosymbionts were synthesizing for their Hemipteran hosts, as well as specific mechanisms of host regulation of symbionts. It was discovered that methionine is the principal amino acid produced by aphid endosymbionts, but symbionts also synthesize sterols required for embryogenesis in many Hemipterans (Douglas 1988a, 1988b, Haas and Konig 1987, Jigami et al. 1986, Lee and Hou 1987, Schwemmler 1987). Mycetocytes were found to vary in both size and number based on the developmental age of aphid host (Douglas and Dixon 1987). It was known previously that symbionts supply their host with nutrients such as cholesterol, certain fatty acids, and certain amino acids, but protein synthesis via symbionts was not well evaluated prior to 1982 (Ishikawa 1984b, 1982b). It was revealed that the aphid endosymbiont exclusively

synthesized one protein, symbionin, critical for cell viability and bacteriophage capsid assembly (Hara et al. 1990, Hara and Ishikawa 1990, Ishikawa 1984b, 1984c, Ishikawa and Yamaji 1985 I and II). Some leafhopper endosymbionts were found to synthesize proteins at very low levels (Douglas 1988c). Intracellular symbiotes of the pea aphid did not synthesize linoleic acid (De Renobales et al. 1986). It was also suggested that host control of symbionts occurred through the secretion of low molecular weight substances that inhibit synthesis of macromolecules in the endosymbionts (Douglas 1989, Ishikawa 1984a). Although literature on microbial symbiosis in Hemiptera during the 1980s is rather narrow in focus, the information gained on symbiotic enhancement of host nutrition, the results laid the foundation for future researchers to address specific questions about the molecular biology and evolution of Hemipteran symbionts.

1991-2005

Molecular characterization and genome reduction in endosymbionts

Aphid symbiotic research remained the most extensively studied association, covered broadly by several highly-cited reviews (Baumann 2005, Baumann et al. 1995, Baumann et al. 1997b, Douglas 1998, Moran et al. 2003a). The focus of these prominent publications highlighted the shift from an understanding of the structural and functional aspects of Hemipteran microbial symbiosis towards evaluations of the genomic and evolutionary links between hosts and symbionts. Some reviews focused exclusively on *Buchnera*, mainly on the genetic characterization, physiology, and evolutionary relationship with their aphid hosts, while others delved into the genomic aspects of nutrient provisioning by *Buchnera*.

Nevertheless, research on symbiotic microorganisms expanded further into non-aphid and hopper groups of similar economic importance, especially Aleyrodids, Pseudococcids, and Psyllids (Baumann et al. 2002, Costa et al. 1995, 1993b, 1996, Downie and Gullan 2005, Franke et al. 1999, 2000, Frank-Whittle 2004, Fukatsu and Nikoh 1998, 2000, Hypsa and Aksoy 1997, Hypsa and Dale 1997, Munson et al 1993, Spaulding and von Dohlen, Subandiyah et al. 2000, Szklarzewicz and Moskal 2001, Thao and Baumann 2004a, b, Thao et al. 2004, 2002, 2001, 2000a, b, 1998, von Dohlen et al. 2001). Buchner (1965) first observed the mycetomes of whiteflies as paired oval structures of orange-yellow color, but since endosymbiotic bacteria were largely uncultivable, little else was known of their structure, function, evolutionary association, or similarity to endosymbionts in other plant-feeding Hemipterans. Through 16S rDNA sequence comparisons, the primary bacterial endosymbiont of the whitefly *Bemisia tabaci* was found to be a vertically-transmitted *Gammaproteobacteria* unrelated to aphids and mealybugs, but a secondary symbiont was closely related to aphid secondary endosymbionts (Clark et al. 1992, Costa et al. 1993, Darby et al. 2001). The primary endosymbiont was later determined to be related to the genus *Pseudomonas*, and the secondary symbiont to be *Arsenophonus*, but *Wolbachia* was also commonly found associated with certain haplotypes (Nirgianaki et al. 2003, Thao and Baumann 2004a, b, Zchori-Fein and Brown 2002). Mealybug bacteriomes were found to house two types of endosymbiotic bacteria, a *Gammaproteobacteria* living symbiotically inside the *Betaproteobacteria* in a tripartite symbiosis relationship (Thao et al. 2002, Von Dohlen et al. 2001). Gram-negative bacteria were isolated and observed from the hemolymph, heart, nerve ganglia, salivary glands, muscles, and gonads from *T. infestans*, but

from an unknown taxa (Hypsa 1993). It was hypothesized that these 'endocytobionts' were vertically transmitted. In addition to previous bacterial isolations (Cavanagh and Marsden 1969), the gut fauna of both *R. prolixus* and *T. infestans* was further characterized, and the gut was shown to house *Serratia marcescens* and *Pseudomonas aeruginosa* (Figueiredo et al. 1995). With this knowledge of bacterial symbionts increasing, some researchers posed the questions of the possibilities of exploiting this aspect of their biology for control, and whether symbiotic bacteria or their metabolites have roles in pathogen suppression and modulation of host vector competence (Azambuja et al. 2005, Azambuja et al. 2004, Beard et al. 2002, 2001, 1998, Durvasula et al. 1997).

The evaluation of the specific function of symbionin also continued into the 1990s, and concluded that it functions as a sole benefit to *Buchnera* since it was synthesized by and localized within the endosymbiont, not in the cytoplasm of the bacteriocyte (Fukatsu and Ishikawa 1992a). It is not present in all aphids however, and it, as well as other chaperonins, were eventually determined to be heat shock proteins (Fukatsu and Ishikawa 1992b, 1992c, 1993). Persistent discoveries of nutrient biosynthesis via symbionts also showed that *B. aphidicola* in aphids synthesized tryptophan, which is in high demand in rapidly-growing aphids, as well as riboflavin, both essential nutrients to the host (Baumann et al. 1997a, Douglas 1998, Douglas and Prosser 1992, Lai et al. 1996, 1994, Nakabachi and Ishikawa 1999, Rouhbakhsh et al. 1996). However, interclonal variation in tryptophan production does occur in aphid populations, which is hypothesized as a correlation to observed differences in nutritional physiology and performance (Birkle et al. 2002, Sandström and Pettersson 1994). It was also found that the pea aphid mycetocytes readily took up glutamine from host

hemolymph, converted it into glutamic acid which was absorbed by the symbionts, and utilized as a source of nitrogen to synthesize seven other amino acids (Sasaki and Ishikawa 1995, Wilkinson and Douglas 1996).

With the advent of next generation sequencing technology such as Massively Parallel Signature Sequencing (MPSS), pyrosequencing, and Sequencing by Synthesis (SBS), the early 2000s saw a rapid advancement in detailed evaluations of symbiont genomes. As genomes became easier and less expensive to sequence, significant genome reduction in endosymbionts was discovered. Compared to genomes of other bacterial taxa, the genomes of symbionts such as *Buchnera* were found to be of considerable less size, highly conserved from multiple isolates, and showed evidence of horizontal gene transfer (Gil et al. 2002, Moran 2003, Moran and Mira 2001, Moran et al. 2003, Shigenobu et al. 2000, Silva et al. 2001, van Ham et al. 2003, Wernegreen and Moran 2000, Wernegreen et al. 2000, Zientz et al. 2001). Evidence of this trend of evolutionary factors affecting genome size was discovered for most primary, obligate endosymbionts across insects and arthropods (Baumann 2005, Wernegreen 2002). One of the consequences of a reduced genome is the loss of regulatory capacity. Even though *Buchnera* was shown to contain more than 20 heat stress genes, only five were differentially expressed under extreme temperature treatments, which validated temperature sensitivity in *B. aphidicola* and suggested that the smaller genome compared to *Escherichia coli* exhibited transcriptional inflexibility (Moran and Wernegreen 2000, Wilcox et al. 2003). The characterization of symbiotic microorganisms in other Hemipterans during this period was significant since many groups are serious pests of agricultural commodities and considered economically-important. The discovery of severely

reduced genomes of symbiotic microorganisms offered new insights into the evolutionary relationships bacteria have mended with their hosts, and illustrated the need to explore deeper into the functional genomics of endosymbionts.

Phylogenetic relationships

As research intensified on the phylogenetic relationships of symbionts and their Hemipteran hosts, in insects with nutritionally unbalanced or strict diets, it became more and more evident that this feeding strategy gave rise to evolutionary mutualistic relationships with symbiotic microorganisms. Phylogenetic analyses revealed parallel evolutions and co-speciation between many Hemipteran hosts and endosymbionts (Downie and Gullan 2005, Munson et al. 1991, Spaulding and von Dohlen 2001, Thao et al. 2000a, Wernegreen 2002, Wernegreen and Moran 2001, Wernegreen et al. 2001). Based largely on paleontological evidence, strong support from molecular phylogenetic studies supported the hypothesis that a consistent pattern of parallel cladogenesis existed in the bacteriocyte-associated bacteria and aphids (Moran & Telang 1998). The estimated age of symbiotic association in aphids is among the oldest; the *Buchnera-Melaphis rhois* association is estimated to have established 150-250 million years ago (Martinez-Torres et al. 2001, Moran et al. 1993, Moran & Telang 1998, Munson et al. 1991, Wernegreen et al. 2000).

Endosymbiosis has descended from ancient bacterial infections. Aphids, leafhoppers, treehoppers, planthoppers, cicadas, and spittlebugs were shown to have a common ancestor from the *Bacteriodetes* symbiont clade, where fossil evidence showed diversification dates at least to the Permian era 260-280 million years ago in the *Fulgoromorpha* (Moran and Wernegreen 2000, Moran et al. 2005). The majority of phylogenetic research assigned the

position of respective endosymbionts in eubacterial phylogeny, and illustrated their relationships with other endosymbionts (Clark et al. 1992, Franke-Whittle et al. 2004, Fukatsu and Ishikawa 1996, Moya et al. 2002, Munson et al. 1991, 1993, Noda et al. 1995, Spaulding and von Dohlen 2001, 1998, Thao and Baumann 2004a, 2004b, Thao et al. 2002, 2001, 2000b, Xet-Mull et al. 2004). Several studies, including reviews, specifically addressed the evolutionary relationship between aphids and *Buchnera* alone (Baumann et al. 1997b, 1995, Braendle et al. 2003, Fukatsu 2001, Martinez-Torres et al. 2001, Moya et al. 2002, Munson et al. 1991).

The evolutionary and phylogenetic relationships of endosymbiotic bacteria of aphids and psyllids were evaluated more than any other Hemipteran group from 1991-2005, but three publications gave some attention to blood-feeding true bugs. The phylogenetic characterization of transovarially-transmitted endosymbionts of *C. lectularius* illustrated that the previously-assigned taxon of *Symbiotes lectularius* in 1957 was actually *Wolbachia* (Hypsa and Aksoy 1997). The results from this study also found that a second bacteria in the *Gamma*-subdivision of *Proteobacteria* showed high similarity (>97%) to the bacterial parasite of *Euscelidius variegatus*, and shared the same evolutionary lineage (Hypsa and Aksoy 1997). It was later demonstrated through Bayesian, maximum likelihood, and maximum parsimony algorithms that *Wolbachia* endosymbionts from the common bed bug, as well as the swallow bug *Oeciacus vicarius*, formed a monophyletic group in the F clade that originated from horizontal transfer (Rasgon and Scott 2004). In another hematophagous Hemipteran, an intracellular bacteria previously undescribed but isolated from several tissues, was proposed to be a secondary endosymbiont in *T. infestans* (Hypsa and Dale 1997).

The bacteria was uncultivable in several bacteriological media, and the 16S rDNA phylogenetic analysis showed this bacteria to have high similarity (96.2%) to *Arsenophonus nasoniae*, the son-killing bacteria of *Nasonia* parasitic wasps (Hypsa and Dale 1997). The ambiguous understanding of the mutualistic relationships between host and symbiont and their evolutionary timeline became clearer with each phylogenetic analysis of the respective association. However, research on plant-feeding Hemipterans dominated the literature on phylogenetic relationships, which left researchers poised to further evaluate blood-feeding Hemipterans.

Recent Focus

Publications since 2006

The past 10 years has seen a surge in research on not only in insect-microbial interactions as a whole, but certainly within the Hemiptera. Figure 1 illustrates the number of publications examined relative to this review that clearly elucidates the trend in this area of research over the past 160 years. The surge seen in the literature beginning in the late 2000s that continues to most recently published articles could be due to the initiation of the United States National Institutes of Health Human Microbiome Project (HMP) in 2008. The project likely opened up a plethora of available funds to conduct research in related areas, including insect-microbial interactions, especially in agricultural pests and vectors of human and animal disease. In fact, in 2001, the American molecular biologist Joshua Lederberg first coined the term 'microbiome' to "*signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space*" (Lederberg and McCray 2001). Since

2011 and through the first half of 2016, the number of related publications on the microbial interactions within Hemiptera is already nearing the previous decade's levels, and is on track to surpass the previous decade as research continues to progress through 2020.

Research conducted over the past decade on symbiotic associations in Hemiptera covered many areas, including microorganism community composition and diversity, role of gut microbes, horizontal transfer, facultative symbionts, and symbiont-mediated protection, as well as further exploration into symbiont genome reduction, genome sequencing of endosymbionts, and phylogenetic and evolutionary relationships. Since several exceptional reviews cover the majority of the genetic and evolutionary research, this section of the review will focus on gut microbes, facultative symbionts, evidence of horizontal transfer, symbiont-mediated protection, and microorganism associations in hematophagous Hemiptera. **Table 1.1** illustrates the symbiotic bacterial diversity discovered in several families of Hemiptera, as well as association type, method of acquisition, and reference to publication.

For several decades, researchers have known of the role endosymbionts play in insect nutritional ecology, but within just the past decade, investigators have discovered additional, often multi-functional roles of symbiotic microorganisms that mediate other ecological traits. These additional roles include enhance parasite resistance, facilitate microbial-mediated immunity, enhance heat stress tolerance, support beneficial manipulation of plant physiology, and influence phenotypic variation (Feldhaar 2011). Research has focused heavily on the role of gut microbiota, the influence facultative symbionts have on host biology and ecology, and

on the acquisition of microorganisms through horizontal transfer. Additionally, since 2006, research in the symbiotic relationships of blood-feeding Hemiptera expanded significantly.

Gut microbes

Microbial colonization of the insect gut and the overall influence on host biology has piqued the interest of many entomologists and microbiologists alike in recent years. Insect gut diversity can vary depending on the environmental habitat, as well as diet, life stage, and host phylogeny (Yun et al. 2014). Many Hemipteran insect species harbor obligate endosymbionts in the gut, but several have been found to also house opportunistic and facultative species of microbes that provide beneficial properties. The morphology and function of Hemipteran digestive tracts is also very diverse, as illustrated and described by Engel and Moran (2013), and therefore has a significant impact on the respective microbial community construct. Several species of Hemiptera acquire their gut bacterial symbionts vertically by way of the fecal-oral coprophagy pathway, while others acquire them from the environment. Most Hemipteran insects have only a few microbial species present in the gut, but aphids, bean bugs, certain species of stinkbugs, and blood-feeding reduviids possess large communities of specialized gut bacteria (Engel and Moran 2013). Microbes that colonize the gut must overcome numerous challenges that include unfavorable physiochemical conditions, several digestive enzymes, host immune responses, and physical disturbance (Douglas 2015). In most insects, the hindgut contains the largest microbial populations since the midgut presents a very hostile environment (enzymatic activity, reactive-oxygen species, pH > 3), but in many plant-feeding Hemiptera that have a midgut pH in the 6-7 unit range and lack a

peritrophic membrane, the midgut offers a suitable environment for symbiotic microorganisms, both obligate and facultative (Douglas 2015).

A host of researchers studied the gut microbes of stinkbugs in the past decade; most examined localization within the host, influence on longevity and fecundity, and the various methods of transmission. The polyphagous and cosmopolitan pentatomid stink bug *Nezara viridula* L. was shown to have an obligate association with its *Gammaproteobacterian* symbiont that colonizes the caecal crypts of the posterior midgut (Hirose et al. 2006, Tada et al. 2011). Studies of aposymbiotic *N. viridula* and experiments with surface sterilization of eggs strongly suggested that the bacterium was acquired orally, not via transovarial transmission, and that maintenance of the symbiont was a function of temperature (Prado et al. 2006, 2009, 2010). Sterilized insects showed increased longevity at 20°C, but fecundity was severely affected as no eggs were laid in those treatments (Prado et al. 2009). Conversely, aposymbiotic *N. viridula* reared under 25°C in another study exhibited severe nymphal mortality and reduced adult emergence, which supported the obligatory characteristic of the symbiont, but also suggested that relative abundance of the symbiont may depend on geographic location, temperature, and climate (Tada et al. 2011). Consistent across many other species in Pentatomidae, including the brown-marmorated stink bug *Halyomorpha halys* (Stål), a recent invasive species in North America, symbionts in female stink bugs were harbored in specialized regions of the posterior midgut where several rows of crypts were morphologically distinct and enlarged (Bansal et al. 2014, Hayashi et al. 2015, Kikuchi et al. 2012, Matsuura et al. 2014, Prado and Almeida 2009b, Taylor et al. 2014). Prevention of vertical transmission of gut symbionts onto eggs negatively affected *H. halys*

and the green stink bug *Acrosternum hilare*, where nymphal development time decreased significantly, females showed reduced fecundity and longer pre-oviposition periods, and egg hatch rate was significantly reduced (Prado and Almeida 2009b, Taylor et al. 2014).

However, the extent of mutualism between gut symbionts and pentatomid species is variable; mean generation time was significantly longer and survivorship increased marginally upon elimination of gut symbionts via egg surface sterilization in the harlequin bug *Murgantia histrionica* (Hahn) (Prado and Almeida 2009b). In the family Scutelleridae, which contain significant pest species of wheat and barley, egg surface sterilization also indicated that primary bacterial symbionts played crucial roles in nymphal development (Çelebi et al. 2014, Kafil et al. 2013). The scutellerid *Cantao ocellatus* (Thunberg) and pentatomid coffee pest *Antestiopsis thunbergii* (Gmelin) both harbored additional, perhaps facultative bacterial species in the genus *Sodalis*, the secondary symbiont of the tsetse fly (Kaiwa et al. 2010, Matsuura et al. 2014). Phylogenetic assessments of pentatomid gut symbionts placed them in a well-defined, monophyletic group of the *Gammaproteobacteria* in the *Pantoea* and *Erwinia* clade separate from other stink bugs in Acanthosomatidae, Plataspidae, Parastrachiidae, and Scutelleridae (Bansal et al. 2014, Kikuchi et al. 2012, Matsuura et al. 2014, Prado and Almeida 2009a). The primary gut symbionts of *C. ocellatus* were found to be phylogenetically distinct, with an independent evolutionary origin from all other stink bug groups (Kaiwa et al. 2010). Additional molecular phylogenetic analysis of 124 species of stink bugs from 20 families for the bacterial symbiont *Burkholderia* revealed an ancient, yet infrequent association, a substantial absence of vertical transmission, and the

symbionts were easily cultivable on standard microbiological media, which suggested environmental acquisition of *Burkholderia* (Kikuchi et al. 2011a).

Various other strategies were found to exist across other stink bug families for vertical transmission of symbionts. Non-social plataspid and parastrachiid females were found to possess specialized traits for the production of a 'symbiont capsule' excreted upon oviposition onto or near the egg mass for consumption by nymphs (Hosokawa et al. 2006, 2007, 2010a). Excessive maternal investment in capsule production for symbiont transmission was also discovered. Females produced 1.7 times more symbiont capsules than nymphs required; capsules contained 1.2×10^8 symbionts, but nymphs only possessed 2×10^7 symbionts consistently (Hosokawa et al. 2007). In addition to within midgut crypts of acanthosomatid females, symbionts were also discovered in a pair of 'lubricating organs' associated with the ovipositor for coating the egg surface (Kikuchi et al. 2009). Some species in the sub-social family cydnidae exhibit peculiar parental care of offspring where they not only smear symbiont-containing substances on eggs upon oviposition, but remain in the vicinity of the egg mass and offer symbiont-rich secretions at later stages either just before or after hatching (Hosokawa et al. 2013). Similarly to Pentatomidae, phylogenetic characterization of plataspid, parastrachiid, acanthosomatid, and cydnid stink bug symbionts placed them in the *Gammaproteobacteria*, and strict host-symbiont co-speciation and reductive genome evolution was commonly found. With the exception of the family Cydnidae, where gut symbionts were found to be polyphyletic, gut symbionts typically formed distinct, monophyletic lineages or individual clades closely related to *Buchnera* and

Baumannia symbionts of aphids and sharpshooters respectively (Hosokawa et al. 2006, 2010a, 2012, 2013, Kikuchi et al. 2009).

Upon investigation into the diversity, localization, and transmission routes of gut symbionts in other Hemipteran families, it was clear that a wide diversity of bacterial species and transmission methods existed, and localization within the host lacked consistency even within the same family. Many of these recent studies used fluorescent *in situ* hybridization (FISH) that revealed the locale and morphology of bacterial symbionts. FISH illustrated the presence of the Actinobacterial symbiont *Coriobacterium glomerans* of the red firebug *Pyrrhocoris apterus* L. in the midgut, rectum, and feces (Kaltenpoth et al. 2009). Symbionts were both acquired vertically via egg-smearing by the females, and horizontally through symbiont-coated eggshells, feces, and adults (Kaltenpoth et al. 2009). Despite being an extracellular gut symbiont, through comparative transcriptome analysis, *C. glomerans* exhibited an integral part in the metabolic homeostasis of the African cotton stainer *Dysdercus fasciatus* Sign.; the bacteria was significantly involved in the processing of B vitamins in the pyrrhocorid (Salem et al. 2014). In the family Lygaeidae, FISH revealed that in the bulrush bug, *Chilacis typhae* (Perris), the endosymbionts resided in a structure of circularly-arranged, enlarged epithelial cells at the end of the first midgut section described as a 'mycetocytic belt' (Kuechler et al. 2011). However, in contrast to *C. typhae*, other species within Lygaeidae, as well as in Blissidae, did not harbor their bacterial endosymbionts extracellularly in the gut, but rather intracellularly in bacteriomes; the structure and localization markedly different among species (Kuechler et al. 2010, 2012). In the case of the symbionts of the birch catkin bug, *Kleidocerys resedae*, their

Gammaproteobacteria showed a strong phylogenetic relationship again with the *Wigglesworthia* endosymbiont of the tsetse fly, and were co-infected with *Wolbachia* and *Rickettsia* (Kuechler et al. 2010). *Wolbachia* was also the most common genus isolated from the anterior foregut of the invasive glassy-winged sharpshooter *Homalodisca vitripennis* in the family Cicadellidae, but the phytopathogenic bacterium *Xylella fastidiosa*, the major cause of Pierce's diseases in grapevines, simultaneously colonized the foregut (Hail et al. 2010, Rogers and Backus 2014). The bacterial endosymbiont *Cardinium*, commonly associated with numerous arachnids, has been found in other cicadellids, although not restricted to gut tissues (Marzorati et al. 2006, Sacchi et al. 2008). In Coreidae and Cicadidae, limited, but consistent gut diversity was discovered. *Pseudomonas* and *Enterobacter* dominated the gut of the cicada *Meimuna mongolica*, and in the giant mesquite bug *Thasus neocalifornicus*, *Burkholderia* dominated the midgut bacterial community, but both nymphs and eggs also contained *Wolbachia* (Oliver-Espejel et al. 2011, Zhou et al. 2015).

Burkholderia is also the primary endosymbiont of the bean bug, *Riptortus pedestris*, which is a serious pest of legumes, and resides in the midgut crypts. This mutualistic relationship has been extensively studied in recent years, largely due to its cultivability and genetic manipulability. Researchers discovered that there is a specific developmental window for the environmental acquisition of *Burkholderia*, which must be acquired each generation specifically during the 2nd instar (Kikuchi et al. 2011b). Using green fluorescent protein (GFP) labelled transgenic strains of the symbiont and live-imaging, researchers observed the spatiotemporal dynamics of the actual colonization process. Colonization began at around 6 hours after inoculation, and crypts were fully colonized by 72 hours after inoculation

(Kikuchi and Fukatsu 2014). It was also discovered that efficient colonization can occur from relatively few cells, symbiont infection was developmentally regulated by the host, and *R. pedestris* had a specialized intestinal organ in the middle of the gut that sorted symbionts by selectively allowing specific bacteria to pass (Futahashi et al. 2013, Kikuchi and Yumoto 2013, Kikuchi et al. 2011b, Ohbayashi et al. 2015).

Symbiotic relationships within the gut of Hemipterans remain poorly understood, but continued efforts in research on the influence of gut microorganisms using insects as model systems holds tremendous promise (Engel and Moran 2013, Pernice et al. 2014).

Facultative symbionts

Previous literature has concluded the ancient acquisition and long-term co-diversification of heritable primary symbionts in Hemipteran insects (Baumann 2005, Baumann et al. 2006, 1995, Degnan et al. 2011, Moran et al. 2008, 2005, Moran and Telang 1998, Munson et al. 1991). In addition to primary symbionts, many groups of Hemipterans have been found to harbor facultative symbionts across a wide range of species, though not all species possess secondary symbionts. A great majority of recent literature has focused on microbial community composition and diversity with emphasis on the presence of other bacteria in addition to the primary symbiotic bacterial species, but also towards an increased understanding of the roles facultative microbes play on the biology of their host.

Facultative bacterial mutualists, like primary mutualists, can confer adaptive benefits to their host including protection from natural enemies and heat stress tolerance (Russell and Moran 2006, Zchori-Fein and Bourtzis 2012). Yet, by the nature of their description, facultative symbionts are not required for survival or reproduction, and are mutualistic

ecologically (Feldhaar 2011, Oliver et al. 2010). Many sap-sucking Hemipterans are often found to be infected with multiple types of facultative bacterial symbionts (Curley et al. 2007, Sandström et al. 2001, Zytynska and Weisser 2016). Facultative symbiont communities of host-specialized biotypes of *A. pisum* sampled from nine different plant species in western France differed substantially between but not within biotype, and *Wolbachia*, *Erwinia*, and *Pantoea* were commonly isolated (Gauthier et al. 2015). In central Chile, the most frequent facultative endosymbionts in *A. pisum* on peas and alfalfa were *Hamiltonella defensa* and *Serratia symbiotica*, with *Regiella insecticola* in multiple species of cereal aphids (Sepúlveda et al. 2016). *Serratia symbiotica* appears to have a complementary metabolic role with the primary symbiont *Buchnera* in the cedar aphid *Cinara cedri*. Whole-genome sequencing of *S. symbiotica* from *C. cedri* revealed the facultative symbiont in an evolutionary stage of genome reduction towards a more obligatory role, as the bacteria biosynthesized tryptophan and riboflavin typically done by *Buchnera* (Lamelas et al. 2011). Wulff and White (2015) hypothesized that the ability of the soybean aphid *Aphis glycines* to colonize resistant soy varieties could be due to the presence of facultative *Arsenophonus*, but determined that aphid virulence was independent of infection with the bacteria. Facultative endosymbionts of pea aphids in the genera of *Regiella*, *Rickettsia*, *Rickettsiella*, and *Spiroplasma* offer resistance against the entomopathogen *Pandora neoaphidis* by reducing mortality and decreasing fungal sporulation (Lukasik et al. 2013, Smith et al. 2015). However, elimination of facultative *Rickettsia* had no significant effect on the type or quantity of reproductive morphs produced, sexual or asexual (Simon et al. 2007). Some secondary symbionts such as *H. defensa*, are able to invade naïve aphids, but

unlike its primary symbiont *Buchnera*, is only conditionally beneficial (Degnan et al. 2009, Sepúlveda et al. 2016). They accomplish this primarily through protein secretion systems that translocate macromolecules from the cell cytoplasm to the bacterial surface or extracellular matrix (Dale and Moran 2006). As of 2014, a total of eight secondary symbionts have been described in aphids (De Clerck et al. 2014). Aphid microbial symbiosis, which is considered the model system for studying insect-microbial symbiosis and interactions, facultative symbionts have been shown to have distinct defensive roles, but little is known about the effects of secondary symbionts in aphid field-systems (Oliver et al. 2014, Smith et al. 2015, Zytynska and Weisser 2016). The recent review by Zytynska and Weisser (2016) on the natural occurrence facultative symbionts in aphids provides a comprehensive overview of the literature in this field, and advocate for much more research on the community level and ecosystem effects of secondary symbionts in aphids.

Across numerous surveys and examinations into the spatiotemporal microbial communities of various Hemipterans, *Wolbachia* was one of the most frequently found facultative symbionts. It was identified in psyllids, whiteflies, sharpshooters, planthoppers, and many species of aphids (Arp et al. 2014, Bressan et al. 2009, Chiel et al. 2009, Gauthier et al. 2015, Hail et al. 2012, 2011, Hughes et al. 2011, Qu et al. 2013, Thierry et al. 2011). *Arsenophonus* was perhaps the second most frequently identified secondary symbiont in recent literature. In addition to their primary endosymbiont *Candidatus* Carsonella ruddii, the potato psyllid *Bactericera cockerelli* was found to have a diverse facultative bacterial community, co-infected with multiple combinations of *Wolbachia*, *Pseudomonas*, *Sodalis*, *Rhizobium*, *Mycobacterium*, *Acinetobacter*, *Methylibium*, or *Xanthomonas* across a global

locations (Arp et al. 2014, Hail et al. 2012, Nachappa et al. 2011). Yet, potato psyllids collected from the West coast of the United States contained a less diverse microbial community than those collected from the Central U.S., which presents unique challenges for targeted biological control (Arp et al. 2014, Hail et al. 2012). In populations of the brown planthopper *Nilaparvata lugens* from China and Southeast Asia, the distribution and localization of both *Wolbachia* and *Arsenophonus* as facultative endosymbionts were examined. In the 15 separate populations evaluated, individual planthoppers contained either *Wolbachia* or *Arsenophonus* localized in mycetocytes within the fat body, in addition to their primary yeast-like symbionts (Qu et al. 2013). Hail et al. (2011) conducted a culture-independent survey of the entire microbiome of *H. vitripennis* to identify the major bacterial fauna in a comparison of the gut, hemolymph, and whole insect that revealed the absence of the plant pathogen *X. fastidiosa*, but rather a significant presence of *Pectobacterium* (formerly *Erwinia*) that is known to cause soft rot and black leg in potato plants.

Facultative plant pathogenic bacteria have been isolated from other sap-sucking Hemipterans as well. Microbial symbionts isolated from the pseudococcid mealybug *Planococcus ficus*, a major pest that vectors pathogenic grapevine viruses, were common in field-collected samples, but absent in lab-reared samples (Iasur-Kruh et al. 2015). The researchers suggested further examination into a possible link between these facultative symbionts and the persistence and transmission of grapevine viruses by *P. ficus*. The cixiid planthopper *Pentastiridius leporinus* was found to not only harbor *Wolbachia*, but also the sugar beet pathogen *Candidatus* Arsenophonus phytopathogenicus (formerly SBR *Proteobacterium*) that *P. leporinus* has been known to transmit in Europe (Bressan et al.

2009). Researchers postulate that this shift of *Arsenophonus* from an endosymbiont to phytopathogenicity could be the result of chance meeting of favorable environmental conditions for cixiid transmission to susceptible host plants (Bressan et al. 2012). Low density, cryptic, and variable infection rates of *Wolbachia* in planthopper populations could suggest that *Wolbachia* may be more pervasive than currently accepted, and represent a true reflection of the actual infection frequency (Hughes et al. 2011).

The diversity of facultative bacterial symbionts within the whitefly *Bemisia tabaci* is extensive and varies considerably via geographic location and whitefly biotype (Chiel et al. 2009, Fujiwara et al. 2015, Park et al. 2012, Singh et al. 2012, Skaljac et al. 2010, Thierry et al. 2011). *Wolbachia*, *Arsenophonus*, *Cardinium*, *Rickettsia*, *Bacillus*, *Enterobacter*, *Paracoccus*, and *Hamiltonella* have all been isolated from *B. tabaci*. In a unique, intracellular ecosystem, *Hamiltonella*, *Wolbachia*, *Arsenophonus*, *Cardinium*, and *Rickettsia* shared the bacteriocyte with *B. tabaci*'s primary symbiont *Portiera aleyrodidarum* (Gottlieb et al. 2008). *Cardinium* and *Arsenophonus* may have an influence on the reproduction of indigenous (Ms) and invasive (B) biotypes of *B. tabaci* (Thierry et al. 2011). Furthermore, *Hamiltonella* may play a role as a nutritional mutualist in *B. tabaci*. Researchers discovered that *B. tabaci* reared on a standard nitrogen diet exhibited neither a fitness cost nor benefit when infected with *Hamiltonella*, but showed increased growth rates on low-nitrogen diets compared to uninfected individuals (Su et al. 2014). It is suspected that *Hamiltonella* may synthesize several cofactors and the amino acid lysine in a complementary metabolic system with the primary symbiont *Portiera aleyrodidarum* (Luan et al. 2015). Several facultative, cultivable bacterial species isolated from pentatomids indicated a highly diverse fauna of 11

different families of *Actinobacteria* (Ozsahin et al. 2010, Zucchi et al. 2012). However, the ecological role of these 34 phylotypes is still unknown, but offer new insight into their potential use as microbial control agents.

A large amount of recent research on microbial interactions in Hemiptera has been devoted to expanding our knowledge of the multi-faceted roles that facultative bacterial symbionts assume. While numerous species of facultative bacteria have been isolated and some roles identified, many more of the specific roles at the individual and community level have yet to be clearly defined. Additional definite or even fortuitous influence facultative symbionts confer on individual host or population fitness, either positive or negative, remains vastly unexplored.

Evidence of horizontal transfer

Primary, and some secondary symbionts, are transmitted vertically via maternal means. Examples of horizontal transmission of endosymbionts are sparse and mechanisms are still not largely understood, but facultative symbionts have been shown to be transmitted horizontally primarily via co-feeding on the same plant (Oliver et al. 2010, Darby and Douglas 2003, Russell et al. 2003, Darby et al. 2001, Sandström et al. 2001). There is current evidence to support horizontal transmission in recently-recognized secondary symbionts, as well as alternative mechanisms by which facultative symbionts can be transmitted.

The acetic acid bacteria genus *Asaia* has been found in the cicadellid leafhopper *Scaphoideus titanus* (Marzorati et al. 2006, Sacchi et al. 2008), but only recently it was shown to be transmitted by both horizontal and venereal means (Gonella et al. 2012). In another leafhopper species, the facultative bacterium of *E. variegatus* (BEV) is one of the

few cultivable endosymbionts, is acquired horizontally during feeding, and shares many virulence genes with plant pathogens (Degnan et al. 2011). As stated previously in this review on discussion of phylogenetic relationships, BEV is most closely related to *Wolbachia* found in *C. lectularius*. This close relationship between symbionts of unrelated plant-feeding and blood-feeding insects represents independent, horizontal transmission events between these two distinct groups (Degnan et al. 2011). Ultrastructural and localization analyses of the midgut and hindgut of five cicadellid leafhopper species from laboratory colonies revealed both long, tubular-shaped and rod-shaped spiroplasma-like organisms (Ammar et al. 2011). Although these colonies had been maintained for several years, there was no correlation between the origins, host plants, or taxonomic position of the leafhoppers and respective spiroplasma-like organism morphological type (Ammar et al. 2011).

Sexual transfer of facultative symbionts was discovered in aphids. *Hamiltonella defensa* and *R. insecticola* appeared to be transferred with seminal fluids, but not within sperm cells (Moran and Dunbar 2006). Laboratory results coincided with data from aphid field populations where horizontal transmission of these facultative endosymbionts is frequent. Researchers noted that sexual transmission of *S. symbiotica* did not occur in their experiments, but is equally probable in aphids (Moran and Dunbar 2006). The paternal transmission of endosymbionts coupled with the frequent matings between individuals infected with different symbiont strains could explain coinfection rates and any rapid changes in infection titer due to antagonistic coevolution among symbiont strains (Moran and Dunbar 2006). Aphid parasitoids have also been shown to horizontally transmit facultative

symbionts. Researchers were able to demonstrate for the first time that aphid parasitoid wasps transferred *H. defensa* and *R. insecticola* by way of a 'dirty needle,' sequentially stabbing infected and uninfected individuals with a contaminated ovipositor (Gehrer and Vorburger 2012). It was also speculated that parasitoids that utilize multiple hosts could also potentially transmit endosymbionts between species.

Symbiosis and acquisition of the bacterial symbiont *Burkholderia* has been quite well studied within Hemiptera, especially as previously discussed here with stink bugs in the genus *Riptortus*. Both *R. pedestris* and *Riptortus clavattus* acquire the beneficial *Burkholderia* from the environment each generation (Kikuchi et al. 2007, Kikuchi and Yumoto 2013). However, recent evidence has uncovered similar methods of acquisition in other Hemipterans. In the family Blissidae, the oriental chinch bug *Cavelerius saccharivorus*, a major pest of sugarcane, harbored *Burkholderia* that dominated the midgut crypts similarly found in stink bugs (Itoh et al. 2015). However, there was a lack of phylogenetic congruence between symbiont and host populations, and only 30% of newly hatched nymphs acquired the symbiont vertically. Researchers suggested environmental acquisition of *Burkholderia* in some hosts, and concluded that the mixed symbiont transfer strategy could be an intermediary stage from environmental to exclusively vertical (Itoh et al. 2015). Horizontal transfer of facultative symbionts has been shown to exist in a few Hemipteran groups, but this method of acquisition likely occurs in several other species and should be evaluated. It is equally important if not more so, with respect to pest management strategies, to ascertain the various mechanisms Hemipteran pests use to obtain these microbial symbionts.

Symbiont-mediated protection

Bacteria and other microorganisms have numerous metabolic and biosynthetic capabilities otherwise lacking in insects and thus confer a wide array of benefits to their hosts (Brownlie and Johnson 2009, Moran et al. 2008). The specific role of resident microorganisms as mediators of host immunity and protection has been examined in only a few groups of Hemipteran insects, almost exclusively aphids. It is thought that vertically transmitted symbionts may contribute significantly to host immunity (Haine 2008), but recent research has offered new evidence to support that horizontally transmitted, facultative endosymbionts play a substantial role as well.

One of the most well-known examples of symbiont-mediated protection exists in the pea aphid-symbiont interaction, where both primary and secondary bacterial symbionts have been shown to provide a diverse array of protection against fungal pathogens, parasitoid wasp larvae, and thermal stress. Several studies have previously demonstrated the link between the presence or absence of facultative symbionts, specifically *H. defensa* and *S. symbiotica*, and the variable susceptibility of the pea aphid to predation by parasitoid wasps (Brownlie and Johnson 2009, Degnan et al. 2009, Ferrari et al. 2004, Guay et al. 2009, Haine 2008, Oliver et al. 2008, 2005, 2003), but more recent literature has offered new insights into specific mechanisms, functional genomics, and impact of environmental stresses with respect to symbiont-mediated immune protection. Having undergone significant genome reduction, the genome of *H. defensa* is nearly half the size of its nearest free-living relatives, yet has retained more genes and metabolic pathways for cell structures related to pathogenicity, toxin homologs, plasmids, and phage-derived genes than the obligate symbiont *Buchnera* (Degnan

et al. 2009). It is speculated that the bacteriophage (APSE) in *H. defensa* produces a toxin similar to Shiga toxin to disrupt eukaryotic cellular processes as the symbiont elicits an attack on parasitoid wasp larvae, with no evidence of influence on ovipositional behavior of the parasitoid wasp (Brownlie and Johnson 2009, Haine 2008). Guay et al. (2009) discovered a new facultative endosymbiont termed pea aphid X-type (PAX) in *A. pisum*, and in conjunction with *H. defensa*, found that aphids not only maintained high levels of resistance to abiotic stresses, but exhibited previously unrecorded levels of resistance to parasitoids. In a related study, it was found that selection pressures from natural enemies can facilitate an increase in facultative symbionts in aphids. The frequency of *A. pisum* infected with *H. defensa* increased significantly upon repeated exposure to the parasitoid wasp *Aphidius ervi*, but declined over time in the absence of parasitism (Oliver et al. 2008). The results suggested a possible cost to the aphid of harboring *H. defensa*, and offered an explanation of the intermediate frequencies of *H. defensa* in natural populations (Oliver et al. 2008).

Serratia symbiotica does provide the pea aphid a low level of protection against parasitoids but confers a much greater benefit on its aphid host through defense against environmental heat shock (Brownlie and Johnson 2009, Burke et al. 2010a). Two theories proposed on the specific mechanism by which *S. symbiotica* provides protection from heat stress were either through secretion of protective metabolites not produced by other facultative symbionts, or that symbiont lysis is the key to protection (Burke et al. 2010a). *Serratia symbiotica* significantly affected aphid metabolomics. After heat exposure, *S. symbiotica* titers were rapidly reduced, but few *S. symbiotica* metabolites were influenced by the temperature increase; this suggested that rapid metabolite delivery is accomplished

through symbiont lysis under conditions of heat stress (Burke et al. 2010a). Pea aphids are also protected from heat stress by their primary symbiont *Buchnera*. A recurring, single nucleotide polymorphism (SNP) was discovered in *B. aphidicola* laboratory and field strains that governed thermal tolerances. A heat-sensitive allele was discovered that arises through a point mutation within the transcriptional promoter of *ibpA*, which encodes a universal heat-shock protein (Burke et al. 2010b, Dunbar et al. 2007).

It has been known prior to 2006 that the facultative symbiont *R. insecticola*, commonly found in aphids, provide protection against fungal pathogens such as *Pandora neoaphidis*, but the specific mechanisms still elude researchers (Haine 2008, Lukasik et al. 2013). Lukasik et al. (2013) found that pea aphids obtain protection from fungal pathogens from a far greater assortment of bacterial symbionts than previously known, and not solely from *R. insecticola*. The facultative bacteria in the genera *Rickettsia*, *Rickettsiella*, and *Spiroplasma* reduced aphid mortality, and decreased fungal sporulation on dead aphids; the most significant protective effects elicited by *Rickettsia* and *Rickettsiella* (Lukasik et al. 2013). Vorburger et al. (2010) recommended adding *R. insecticola* to the list of endosymbionts which offer protection against parasitoids upon discovering that an isolate from the endosymbiont from the peach-potato aphid *Myzus persicae* in Australia increased aphid resistance to the biocontrol agent *Aphidius colemani*. However, different strains of *R. insecticola* vary in levels of protection for their aphid host, some offering nearly no protection against parasitoids (Hansen et al. 2012). The diverse protective benefits of aphid facultative endosymbionts was revealed in another recent study. Pea aphids co-infected with *Spiroplasma* and one of three strains of PAX were protected from *Pandora neoaphidis* by all

three strains of PAX, and two of the strains increased the resistance to parasitoid wasps (Heyworth and Ferrari 2015). The presence of PAX also increased reproduction in the aphids upon being heat-stressed (Heyworth and Ferrari 2015).

Although insects and invertebrates do not possess the antibody-based, protein-mediated adaptive immune system typical of higher trophic organisms, a few can produce diverse, polymorphic peptides or proteins as a response generated to microorganisms (Nyholm and Graf 2012). Memory-like immunity and the ability to recognize self vs. non-self in insects is largely unknown, but adaptive immune effectors have been identified in *D. melanogaster* and *Anopheles gambiae* (Nyholm and Graf 2012). When the genome of *A. pisum* was finally sequenced, it revealed a significant loss of immune-related genes typically found in other insects, and a simultaneous increase in genes involved in the production of short, non-coding RNAs (Pinheiro et al. 2015). Additionally, in terms of pattern recognition receptors for microorganism-associated molecular patterns (PRR-MAMP), *A. pisum* was found to have a non-functional immune deficiency (IMD) signaling pathway and no peptidoglycan recognition proteins (PGRPs), but Gram-negative binding proteins (GNBPs), a Toll-like signaling pathway, and a haemocyte response were present (Douglas et al. 2011, Nyholm and Graf 2012). With respect to humoral components of *A. pisum*'s innate immunity, both complement-like factors and reactive oxygen and nitrogen species were present, but antimicrobial peptides (AMPs) were reduced (Nyholm and Graf 2012). When the interaction between *B. aphidicola* and non-host, cultured *Drosophila* S2 cells was investigated, *B. aphidicola* induced an immune response, which supported the hypothesis that the absence of PGRPs and a functional IMD signaling pathway in the pea aphid was due to the evolutionary

contribution of the symbiont to the reduced immune capabilities of its host (Douglas et al. 2011, Nyholm and Graf 2012). Also, pea aphids were found to have varying numbers of haemocytes and a variation in encapsulation response depending on the type of facultative symbiont present, *S. symbiotica*, *H. defensa*, or *R. insecticola*, which suggested an influence on cellular immunity by specific symbiont (Laughton et al. 2016, Nyholm and Graf 2012).

It has been proposed by a few investigators that the abundant protein symbionin (now *GroEL*) produced by *Buchnera* in the pea aphid has protected ingested plant viruses from degradation and thus facilitated their spread. Yet, despite numerous studies since the early 1990s, results have failed to prove or disprove *Buchnera*'s involvement in luteovirus transmission, and more research is necessary to elucidate the protein-protein interaction between viral coat proteins and symbiont-produced proteins (Pinheiro et al. 2015). Vertically transmitted symbionts have smaller genomes than environmentally transmitted bacterial symbionts. How hosts respond and adapt to bacterial endosymbionts with various genome sizes is still a mystery (Nyholm and Graf 2012). Fischer et al. (2015) discovered that volatile semiochemicals from *Staphylococcus xylosus* in honeydew of the black bean aphid *Aphis fabae* Scopoli attracted ant scouts and thus enhanced the well-studied ant-aphid mutualistic relationship. This phenomenon deserves a much more in-depth evaluation. Lastly, the specific metabolic mechanisms that allow the insect's beneficial microbiome to elicit or augment an immune response to xenobiotics, yet evade host, non-microbial immune attack is an area currently being investigated. Some have referred to this phenomenon as host tolerance (Weiss and Aksoy 2011). It is proposed that host tolerance either involves symbiont suppression of host immune system functions as a form of self-defense, or host recognition

of molecular signals on foreign bacterial cell surfaces, such as the one mediated by the peptidoglycan (PGN) structure found in bacterial cell membranes (Weiss and Aksoy 2011). However, obligate and facultative endosymbionts have PGN as well, and not all organisms may have PGRPs that would recognize PGN as part of the initial component of the signal transduction pathway, as in the *A. pisum* example (Weiss and Aksoy 2011).

A handful of studies have examined microbial-mediated immunity and the effects of symbionts as a defensive posture in Hemipterans other than aphids. The parasitoid wasp *Eretmocerus mundus* Mercet is a biological control agent against *B. tabaci*, to which the whitefly initiates an immune response. In addition to upregulation of a consortium of host genes for defensive immune pathways and induction of a melanization cascade, a second set of bacterial symbiont genes were highly expressed in *B. tabaci* upon parasitization (Mahadav et al. 2008). The qPCR and FISH results illustrated proliferation of facultative *Rickettsia* that was induced upon initiation of parasitization, which suggested involvement in host resistance to the parasitoid (Mahadav et al. 2008). Bauer et al. (2014) evaluated the molecular relationship between host immune system and the primary symbiont of the cotton stainer *D. fasciatus* through comparative transcriptomics in aposymbiotic individuals and revealed constitutive expression of transcripts for immune signaling pathways, but differential expression of antimicrobial peptides. The findings of a highly differentiated response to the pathogenic-beneficial microbial complex offered strong support that the host immune system serves as a communication interface between host and symbionts (Bauer et al. 2014). In blood-feeding Hemipterans, the hemolytic activity of the facultative symbiont of *R. prolixus* by *S. marcescens* against trypanosomes has been extensively evaluated. Although multiple

strains of *S. marcescens* examined lysed erythrocytes, only the prodigiosin-producing RPH and SM365 strains of *S. marcescens* destroyed the *T. cruzi* Y strain through rapid attachment of numerous bacteria to the epimastigote membrane (Garcia et al. 2007). However, no strain of *S. marcescens* has been found to destroy the DM28c clone of *T. cruzi*, and infection of *R. prolixus* showed that DM28c modified the host gut immune response and decreased the gut microbial population (Castro et al. 2012, Garcia et al. 2007). Infection of *R. prolixus* with *Trypanosoma rangeli* resulted in efficient colonization of the midgut that altered the community microbiota, enhanced midgut antimicrobial peptides against *S. marcescens*, and downregulated lysosomal and defensin activity against the parasite (Vieira et al. 2015). The successful establishment of parasite infection in the gut suggested an immune system modulation of *R. prolixus* by the trypanosome, illustrated the complexity of these parasite-vector-microbiota relationships, and demonstrated the need for additional research into symbiont-mediation protection mechanisms in hematophagous insects.

With recent developments, knowledge of the diversity of endosymbionts that offer protection to their Hemipteran hosts expanded, but the research has been limited to nearly entirely aphids. There are likely numerous other examples of symbiont-mediated protection awaiting discovery, and more in-depth studies of hematophagous as well as phytophagous Hemiptera is needed. Furthermore, many of the specific mechanisms primary and secondary endosymbionts use to confer resistance and protection remain unknown, and the foundation of recognition of self vs. non-self as it applies to symbiotic and xenobiotic microbes is yet to be solidified.

Microorganisms associated with hematophagous Hemiptera

Despite the abundance of research on endosymbiotic relationships and microorganisms found in phytophagous Hemiptera, in the past ten years, researchers have advanced our knowledge of the host-symbiont and resident microorganism associations in the obligate blood-feeding groups of Cimicidae and Reduviidae. Bed bugs have a long and ancient history with humans, and infestations were once thought to be considered as a result of poor hygiene or primarily restricted to developing countries (Sakamoto and Rasgon 2006). However, many experts believe that since the ban on the use of DDT in the U.S. in 1972, pyrethroid resistance, and increased global travel have facilitated the profound resurgence of bed bug infestations all across the world, including developed nations. In the same respect, the threat of Chaga's disease to the U.S. from reduviid bugs such as *R. prolixus* and *T. infestans* has been of considerable concern in the past few years. Research into bed bug biology, physiology, and alternative control methods has increased dramatically over the last 20 years due to the resurgence and difficulty of infestation management. Both of these blood-feeding groups harbor obligate endosymbionts, and perhaps similar to previous hopes of exploiting endosymbionts of plant-feeding Hemipteran pests as a potential means for control, the same premise likely prompted the exploration into comparable novel control methods of bed bugs and kissing bugs.

In the late 1990s, researchers confirmed *Wolbachia* as the primary endosymbiont of the human bed bug *C. lectularius*. However, estimates of the distribution of *Wolbachia* in *C. lectularius* or other species within Cimicidae were unknown at that time, although *Wolbachia*

was estimated to be in as high as 70% of known arthropods (Sakamoto and Rasgon 2006, Sakamoto et al. 2006). In one study, specific PCR primers were used to survey *Wolbachia* infection frequencies in museum specimens of 39 different species of cimicids where infections were determined in approximately 23% of species examined (Sakamoto et al. 2006). Upon further investigation into the geographic distribution of *Wolbachia* in *C. lectularius*, no significant differences in infection frequency were found between geographic location, sex, or life stage, which indicated *Wolbachia* was likely present in all populations (Sakamoto and Rasgon 2006). It was proposed that *Wolbachia* could serve as a target for alternative control measures against bed bugs, citing the cytoplasmic incompatibility, or male-killing phenomenon illustrated in other *Wolbachia*-infected insects (Sakamoto and Rasgon 2006). The specific effect and influence of *Wolbachia* on bed bug biology was not known until a few years later. Confirming the work of Buchner in 1920 and Arkwright et al. (1921), Hosokawa et al. (2010b) used FISH to brilliantly illustrate the localization of *Wolbachia* in several tissues, structures, and life stages of *C. lectularius* and found the highest concentrations in the bacteriomes of both males and females. An additional, perhaps secondary endosymbiont in the *Gammaproteobacteria* classified as an unknown *Enterobacteria* species was also identified (Hosokawa et al. 2010b). Yet, perhaps an even more significant confirmation in the same study of the influence of *Wolbachia* was that the endosymbiont provided B vitamins to its host, as discovered similarly in *R. prolixus* in the 1940s (Hosokawa et al. 2010b). Bed bugs were fed blood meals inoculated with the antibiotic rifampicin to eliminate *Wolbachia*, and the treated groups displayed delayed growth and sterility, but recovered when supplemented with commercial B vitamins (Hosokawa et al.

2010b). When the genome of *Wolbachia* from *C. lectularius* (*w*Cle) was sequenced, a single gene cluster that encoded the complete synthetic pathway for biotin (vitamin B7) not found in other *Wolbachia* genomes was discovered (Nikoh et al. 2014). The presumed acquisition of the biotin gene cluster through lateral gene transfer from co-infected *Cardinium* or *Rickettsia* bacteria offered additional support of *Wolbachia* as an evolutionary mutualist (Nikoh et al. 2014). Together, the results from these two pioneering studies confirmed *Wolbachia* as a bacteriocyte-nutritional mutualist that evolved from a once free-living, facultative association. Recent follow up work on *Wolbachia*'s fitness contribution to *C. lectularius* has revealed a previously unrecognized synthesis pathway for riboflavin (vitamin B2). Researchers examined several insect-associated strains of *Wolbachia* and through the comparative genomic survey for synthetic capability of B vitamins concluded that only the synthesis pathway for riboflavin was highly conserved (Moriyama et al. 2015). Riboflavin-provisioning from *Wolbachia* was found to be as equally vital as biotin to the overall health of *C. lectularius*, as it contributed significantly to growth and reproduction (Moriyama et al. 2015). The results also elucidated that the riboflavin synthesis genes have been stably maintained throughout the course of *Wolbachia*'s evolutionary history.

Other recent genetic analyses of symbionts and resident microorganisms within *C. lectularius* has revealed some peculiar and potentially useful information about their individual and community biology. Moriyama et al. (2012) conducted transcriptomic expressed sequence tag (EST) analyses of the bacteriome and spermalege of both male and female *C. lectularius* and found several highly-expressed antioxidant genes and genes encoding for cysteine proteases such as cathepsins within the bacteriomes. Lipid transport

genes were also abundant, where in each of the bacteriomes, female whole body, and spermalege, ESTs for apolipophorin III were found. The prolixicin antimicrobial peptide gene, actin gene, and two genes encoding actin regulator proteins were also highly-expressed in the spermalege. These highly-expressed ESTs indicate a robust collection of genes and proteins involved in immune response functions. Cathespins belong to the lysosomal proteolytic system, the apolipophorin III protein binds to the surface of microorganisms to stimulate an immune response, and actin genes are highly involved in the cellular immune response against microbes (Moriyama et al. 2012). This information may offer clues or partial explanations as to why bed bugs have not been implicated in the biological transmission of disease; the bed bug microbiome may have an influence on the insect's vector competence. The results of the *C. lectularius* genome sequence project were released very recently (Benoit et al. 2016). Sequencing and gene annotation revealed the presence of many candidate lateral gene transfers from bacteria, including *Arsenophonus*, *Wolbachia*, *Sodalis*, *Hamiltonella*, *Serratia*, *Peptoclostridium*, and a host of others. A nearly complete genome of *Staphylococcus* was also found, which showed high similarity to the bacterium *S. xylosus* (Benoit et al. 2016). Additionally, the bacterial scaffold with strong homology to the bacterium *Pectobacterium carotovorum* (formerly *Erwinia* genus), a common plant bacteria in the Enterobacteriaceae family, was identified, but did not contain a ribosomal locus, and therefore not likely to be an endosymbiont of *C. lectularius* (Benoit et al. 2016).

Surveys of microbes associated with bed bugs at the individual and community level revealed sparse diversity of bacterial species, but also yielded unusual or unexpected results in some cases. Saenz et al. (2013) screened 331 bed bugs from 29 different geographic

locations across 13 states for the re-emergent bacterial pathogen *Bartonella*. Although no *Bartonella* DNA was amplified from any bed bug specimen, five specimens from four different apartments in a multi-unit, elderly housing complex in Raleigh, North Carolina were found to contain *Burkholderia multivorans*. *Burkholderia multivorans* is commonly associated with nosocomial infections such as cystic fibrosis, and was not previously linked to an arthropod vector (Saenz et al. 2013). In the interest of the core community microbiome, and microbial communities that *C. lectularius* could transport, Meriweather et al. (2013) surveyed 31 individual bed bugs from 8 loci around the Cincinnati, OH metropolitan area, which was labeled one of the most bed bug infested cities in the United States at that time. Researchers crushed whole insects and used a culture-independent 16S rDNA method coupled with the longer-read, 454 Titanium pyrosequencing platform to characterize the bed bug microbial diversity, assess whether *Wolbachia* and the unknown *Gammaproteobacteria* previously identified would be found at all sample locations, and potentially determine the presence of any human pathogenic bacteria from residences (Meriweather et al. 2013). The results showed that 97% of the microbial community was dominated by two operational taxonomic units (OTU), *Wolbachia* and the unidentified *Gammaproteobacteria*, with *Wolbachia* at a 63% relative abundance, and the unknown *Enterobacteria* species at 33% (Meriweather et al. 2013). However, *Pseudomonas*, *Sphingomonas*, *Shigella*, and *Methylobacterium* were also identified, but in percent relative abundance < 1%. Whether these low abundant bacterial species were external, surface microbes or colonized the bed bug's gut is undetermined. A similar study was conducted in North Carolina in 2015, but examined the bacterial fauna of the gut of *C. lectularius*. Fisher et al. (unpublished results)

found an appreciable amount of bacterial diversity associated with the bed bug gut, with *Wolbachia* and the unknown *Enterobacteria* species again the most abundant species, but other bacterial species not previously associated with bed bugs. Bacteria commonly found on human skin and in the oral cavity, bacteria known to degrade insecticides, thermophilic bacteria, and other presumably facultative bacteria were found at low (<1%) relative abundance (Fisher et al., unpublished results).

Recent research into the microbial interactions of reduviids also yielded curious results. As mentioned in the previous section on symbiont-mediated protection, both *T. cruzi* and *T. rangeli* have demonstrated the ability to modulate the immune system and gut microbiota of *R. prolixus* (Castro et al. 2012a, Vieira et al. 2015). Castro et al. (2012b) investigated the tripartite relationship between *R. prolixus*, gut microbiota, and *T. cruzi* further to assess the effects of the natural, secosteroid physalin B on parasite survival and microbiota homeostasis. Oral, topical, and contact treatments of physalin B significantly reduced the number of DM28c clone of *T. cruzi* while simultaneously facilitating an increase in the number of gut bacteria (Castro et al. 2012b). The investigators proposed the potential use of physalin B to control trypanosomes in the fight against Chagas disease. While it is known that several species of Triatomine bugs are vectors of trypanosomes, the predominant gut microbiota shows considerable diversity between species, but limited diversity characterized by *Enterobacteriaceae* within individuals of the same species. The microbial composition of the Triatomine vectors *Rhodnius*, *Triatoma*, *Panstrongylus*, and *Dipetalogaster* from Brazil was compared using culture-independent analysis of 16S rDNA and denaturing gradient gel electrophoresis (DGGE) in two recent studies (Da Mota et al. 2012, Gumiel et al. 2015). The

results showed that *Serratia* dominated the gut of *R. prolixus* and *Rhodnius neglectus*, *Arsenophonus* dominated the gut of *T. infestans*, *Triatoma vitticeps*, and *Panstrongylus megistus*, while the gut of *Dipetalogaster maximus* was dominated by *Candidatus* Rohrkolberia/Pectobacterium (Da Mota et al. 2012). *Serratia marcescens* was present in *Triatoma brasiliensis* and *Triatoma pseudomaculata* as the dominant bacterial species, but the genera *Dietzia*, *Gordonia*, *Corynebacterium* were also identified (Gumiel et al. 2015). *Firmicutes* and *Bacteriodetes* were also detected, but in significantly lower percent relative abundance (Gumiel et al. 2015). Since the microbiota of the gut of Triatomines has an integral role in the maintenance and transmission of trypanosomes in this group of Hemipterans, these two studies revealed new information of the microbial composition that is important for design of future management strategies of these pests. One recent study proposed the use of systemic RNAi to modify the gut microbiota in *R. prolixus* as a novel means for paratransgenic control of the vector. Taracena et al. (2015) fed *R. prolixus* nymphs and adults *E. coli* modified to express dsRNA for the Rhodnius-heme-binding protein (RHBP) and catalase (CAT), two genes involved in oxidative stress, and found RHBP and CAT expression was reduced by 96-99% in various tissues. The gene knockdown had deleterious effects on the insect's fitness; mortality rates increased, molting rates reduced by 100%, and oviposition was reduced by 43%. Researchers discussed the potential use of this RNAi delivery system using the primary symbiont *Rhodococcus rhodnii*, and the need for further studies on this system (Taracena et al. 2015).

While numerous recent evaluations of microbial symbiosis and microbial community composition in hematophagous Hemipterans have uncovered previously unknown

information, the presence and spread of bed bugs and Triatomine vectors of Chagas continues. This new, highly-relevant information on what microbes are associated with these blood feeders, and how they influence their biology, can potentially allow us to enter a new realm of management tools to add to our existing integrated pest management toolbox.

Future Directions

The literature over the past 150 years is overwhelmingly dominated by research on three distinct groups of the Hemiptera: aphids, planthoppers, and leafhoppers (Baumann et al. 2006, Baumann 2005, Moran and Telang 1998, Baumann et al. 1997, Baumann et al. 1995, Houk and Griffiths 1980, Buchner 1965). This leaves enormous potential for research in aquatic, predaceous, and hematophagous Hemipterans to greatly improve our understating of the insect-microbe interactions in these other groups. Insects evolve and adapt quickly, as demonstrated through insecticide resistance in several notable agricultural pest species. Hemipteran pests will continue to plague humans as blood-feeding nuisances of human dwellings, vectors of plant and animal diseases, and economically-important damagers of agricultural crops, and thus studying insect microbiomes holds tremendous potential value as an untapped resource for manipulation to control insect pests. The exploitation of the host-symbiont link as an effective and alternative management strategy, defined as 'Microbial Resource Management' (MRM) (Crotti et al. 2012), has been proposed by several researchers. Suggested mechanisms to target include paratransgenesis or inserting foreign DNA through transgenesis of the symbiont (Beard et al. 2002, Douglas 2007, Durvasula et al. 1997, Rio et al. 2004, Taracena et al. 2015), cost-effective disruption of the vertical

transmission process without the use of antibiotics (Douglas 2015, 2007, Prado and Zucchi 2012), and interference between insect and symbiont nutrient translocation (Douglas 2015). The arsenal of chemical and non-chemical control measures for pest management grows limited each year, and as insecticide resistance increases, the need for an enhanced knowledge of insect-microbe interactions is vital to develop novel methods of control, as well as increase our understanding of insect biology.

Symbiosis in aquatic Hemiptera

Very little is known with regard to what symbiotic microorganisms are present in aquatic Hemiptera, and certainly with respect to their function. *Wolbachia* was found via PCR in a species of *Gerris* water striders, but in no other aquatic Hemiptera examined in the study (Werren and Winsor 2000). Insects in aquatic systems are commonly used to assess overall health of that ecosystem through abundance and distribution of insects in the orders Ephemeroptera, Plecoptera, and Trichoptera, known as the EPT index. Since it is likely that resident microorganisms reside in insects from these groups as well, the entire genetic material landscape of the respective environment, including these microorganisms, could be monitored through metagenomics, or community genomics, as an additional method of assessing aquatic ecosystem health. Also, the diversity of gut microorganisms can be dependent on the host environment. There is a distinct contrast among percent relative abundance of gut microbiota, anaerobes vs. aerobes, of flying insects compared to predominantly aquatic insects (Yun et al. 2014). The gut bacteria of insects inhabiting sky or ground habitats are dominated by aerobic microbes, whereas insects found in aquatic environments have more anaerobic microflora. This field of study is wide open for aquatic

and semiaquatic Hemiptera such as those in Nepidae, Belostomatidae, Gerridae, Notonectidae, Pleidae, Saldidae, Naucoridae, and Corixidae. Even though this group is generally not considered pestiferous in agriculture or human and animal health, many aquatic insects consume bacteria-laden detritus of plants and animals. This invites the need for future research into the discovery of microbial taxonomy associated with aquatic Hemiptera, as well as any fitness benefits they are conferring on their hosts.

Symbiosis in predaceous Hemiptera

Of the more than 500 publications examined for this review, only four were found to discuss symbionts or microbial interactions in predaceous Hemipterans. Terrestrial predaceous Hemipterans include assassin bugs, ambush bugs, damsel bugs, minute pirate bugs, and spined soldier bug in the families Reduviidae, Phymatidae, Anthocoridae, Nabidae, and Pentatomidae respectively. The minute pirate bug *Orius laevigatus*, was found to harbor *Wolbachia*, but not the bacterial symbiont *Cardinium* (Zchori-Fein and Perlman 2004). Commercially-available predaceous mirids are currently used in biological control that are natural enemies of aphids, whiteflies, and thrips. *Macrolophus caliginosus*, *Macrolophus pymgaeus,* and *Nesidiocoris tenuis* contain multiple endosymbionts of *Alpha*-and *Gammaproteobacteria*, including *Wolbachia* and *Rickettsia* (Caspi-Fluger et al. 2014, Machtelinckx et al. 2012). However, *Wolbachia* induces severe cytoplasmic incompatibility in its *M. pymgaeus* host, and has perhaps contributed to species formation in the genus *Macrolophus* which may limit its practical use in augmentative biological control (Machtelinckx et al. 2009). High amounts of *Rickettsia* localized in the gut of *N. tenuis*

suggest a nutritional role (Caspi-Fluger et al. 2014). Exploration into symbiotic relationships in predaceous Hemiptera certainly deserves more attention.

Relationships between facultative and obligate symbionts

Continued exploration of the biological interactions between primary, as well as secondary (facultative) symbionts and Hemipteran hosts in co-infected individuals is important for evolutionary and phylogenetic studies. In recent years, the concept of a 'holobiont' has taken shape, as a result of advancements in molecular techniques and an increase in our overall understanding of how microorganisms affect their macroorganism hosts. The emphasis has shifted from studying the form and function of host individuals as a derivative based on genotype and phenotype to a more holistic view of a host as a holobiont, *e.g*. the collective host organism genome and all symbiont genomes regulate host phenotype (Smith et al. 2015, Feldhaar 2011, Rosenberg et al. 2010). Therefore, since symbiotic microorganisms typically have a measurable impact on host fitness and selection can act on phenotypic variation, the holobiont should be regarded as the unit of selection (Feldhaar 2011).

Additionally, the role of facultative symbionts as immune modulators needs to be studied further. A recent study demonstrated the precision of the pea aphid immune response. In the study, the characterization of the immune response of *A. pisum* was measured by examining the differences in immune gene expression when challenged with facultative *S. symbiotica* and pathogenic *S. marcescens* (Renoz et al. 2015). Ingestion of *S. symbiotica* did not affect host survival and colonized the entire alimentary canal within a few days, but 100% mortality was observed after ingestion of *S. marcescens* over the same period, despite the aphid's lysosomal and Jun-N-terminal kinase (JNK) signaling pathway immune response (Renoz et

al. 2015). This example illustrates the aphid's ability to recognize beneficial microbes from harmful, pathogenic species, but specific mechanisms and involvement of other immune responses is unknown. Very little is known about what other groups of Hemiptera harbor *Asaia* or other acetic acid bacteria, so this area of facultative symbiosis is also wide open.

Further research into community level analyses on both obligate and facultative symbionts in Hemipterans is needed due to the fact that symbiont polymorphism exists in many sap-feeding groups, and these groups do not respond to selection pressures in the same way (Zytynska and Weisser 2016). Large variations in selection pressures from natural enemies across seasons in aphids suggests that symbiotic communities are dynamic, and the functional role of the entire community needs further study (Zytynska and Weisser 2016, Smith et al. 2015). The ratio of primary and secondary symbionts has also been shown to be dynamic at the individual level. Under different host-plant induced and xenobiotic stressed conditions, the microbiome of the soybean aphid *Aphis glycines* changes; the ratio of obligate to facultative endosymbionts is dynamic as the percent relative abundance fluctuates with both positive and negative correlations (Enders and Miller 2016).

Microbial-mediated immunity

Although *Wolbachia* has been thoroughly studied in numerous insects, further exploration of the function or perhaps multi-functional role *Wolbachia* plays as an endosymbiotic resident in Hemiptera is warranted. *Wolbachia* is rarely found in aphids, but has recently been consistently found in the banana aphid *Pentalonia nigronervosa* as a facultative symbiont (De Clerck et al. 2015, De Clerck et al. 2014). Its consistent presence in *P. nigronervosa* across several geographic locations in Africa and large number of shared genes

for essential nutrient production, suggest a co-obligatory role with *Buchnera* (De Clerck et al. 2015, De Clerck et al. 2014). While *Wolbachia* exists as an obligate, nutritional mutualist in bed bugs, it may have additional roles in this group, as found similarly elsewhere in mosquitoes and fruit flies. Bed bugs have still not been conclusively linked as disease vectors of any pathogen, and a more definitive answer as to why they have not is still undetermined. In nearly every other blood-feeding arthropod or insect (mosquitoes and biting flies, fleas, lice, ticks, mites) in the medical and veterinary entomological literature, a single pathogen and often multiple pathogens have been associated with these groups of organisms. Yet the common bed bug, strictly by its blood-only diet and close proximity with humans affords the opportunity to acquire and potentially maintain viable blood-borne pathogens. Only very recently have bed bugs been shown to experimentally acquire and maintain *Trypanosoma cruzi* (Chaga's disease) and *Bartonella quintana* (Leulmi et al. 2015, Salazar et al. 2014) experimentally in laboratory settings. It is reasonable to postulate that similar to the symbiont-mediated defense system in aphids, *Wolbachia* antiviral protection in *Drosophila melanogaster*, or the hemolytic attack on erythrocytes infected with *T. cruzi* by the bacteria *Serratia marcescens* in the gut of *R. prolixus*, endosymbiotic *Wolbachia* or other resident bacterial species may confer some type of microbial-mediated immunity to ingested pathogens in bed bugs (Brownlie and Johnson 2009, Azambuja et al. 2004, Garcia et al. 2007, Haine 2008, Hedges et al. 2008, Lukasik et al. 2013, Oliver et al. 2008, 2005, 2003, Degnan and Moran 2008).

One potential huge benefit to in-depth examinations of microbial-mediated immunity and symbiont-mediated defense complexes in insects could yield new weapons in the realm of

antibiotics. In 1928, the Scottish scientist Alexander Flemming (1881-1955) discovered the first natural antibiotic penicillin that formed the basis for a revolution in modern medicine (Kaltenpoth 2009). A large number of bacterial symbionts of insects belong to the *Actinobacteria* Phylum, and approximately 60% of antibiotics currently isolated are produced by *Actinobacteria* (Bode 2009, Kaltenpoth 2009). Many ant and beetle species have symbiotic *Actinobacteria* that produce antifungal compounds, but the mechanisms in many other insect examples is still unknown. In modern human medicine, we are at the doorstep of a new era of multidrug resistant bacteria and pathogens. The hidden potential of antimicrobials derived from insect or arthropod symbiotic bacteria is vastly unexplored. Most symbiotic organisms produce secondary metabolites, and several novel compounds with antimicrobial activity have been isolated (Bode 2009, Kaltenpoth 2009, Piel 2009, Piel 2004). However, this area deserves a concerted effort into exploration of new biomolecules that could potentially allow us to remain one step ahead of antimicrobial resistance in bacteria across the globe.

Symbiont-mediated insecticide resistance

Insecticide resistance mechanisms and detoxification of xenobiotics by insects have been studied for decades, but literature on the role of bacterial symbionts in insecticide resistance is scarce. In the last ten years, however, within the Hemiptera, a few researchers have brought this unique biological relationship to our attention. *Bemisia tabaci* has been the primary insect of concern in these studies, but the bean bug *R. pedestris* has also been identified as exhibiting symbiont-mediated resistance to insecticides. Ghanim and Kontsedalov (2007) identified 111 expressed sequence tags associated with resistance and

xenobiotic detoxification were differentially upregulated in a pyriproxyfen-resistant strain of the Q biotype of *B. tabaci*, and later discovered that the presence of high bacteria densities of *Rickettsia* correlated to the insect's ability to detoxify insecticides (Ghanim and Kontsedalov 2009). However, another study by the same group found conflicting results wherein a *Rickettsia*-free line of *B. tabaci* was 15-fold more resistant to pyriproxyfen than the *Rickettsia*-infected line (Kontsedalov et al. 2008). Kikuchi et al. (2012) investigated symbiont-mediated insecticide resistance of *R. pedestris* in Japan to fenitrothion. The fenitrothion-degrading bacteria *Burkholderia* are found in low densities in agricultural fields, but researchers found that of the bean bugs living on sugarcane, about 8% harbored the fenitrothion-degrading *Burkholderia*. These results indicated a specific and beneficial symbiosis between microbe and host that conferred resistance to fenitrothion (Kikuchi et al. 2012). As more knowledge is gained from studying insect symbiosis and the influence microbes have on host biology, it is likely that more examples of insecticide resistance mediated by microbial symbionts will be discovered. This is a unique area that warrants considerable attention, especially since increased resistance to available chemicals is a significant concern for pest management and sustainability.

Concluding Remarks

In 1998, Angela Douglas, who has devoted more than 30 years to the study of obligate intracellular symbioses, estimated that endosymbiotic relationships with microorganisms exist in perhaps as much as 15% of all insect species (Douglas 1998), but it is quite possible that that number is highly conservative. Based on current and emerging knowledge, and the

number of insect groups yet to be examined, it is more likely closer to 50%. Most endosymbiotic bacteria are not cultivable on common laboratory rearing media, as early investigators discovered again and again, and so their characterization was delayed until the advancement of recombinant DNA methods and sequencing methods such as Sanger and high-throughput were available. Today's sequencing technology and rapid advancements in the molecular field can provide enormous amounts of data on how microorganisms affect their macroorganism hosts, especially in insects and arthropods. The diversity of the form and function of symbiotic microorganisms in insects is remarkable. Yet we have merely explored the tip of the tip of the iceberg with respect to insect-microbial interactions, and as exciting as this field is today, what lies just beyond the horizon awaiting discovery is truly fascinating to contemplate.

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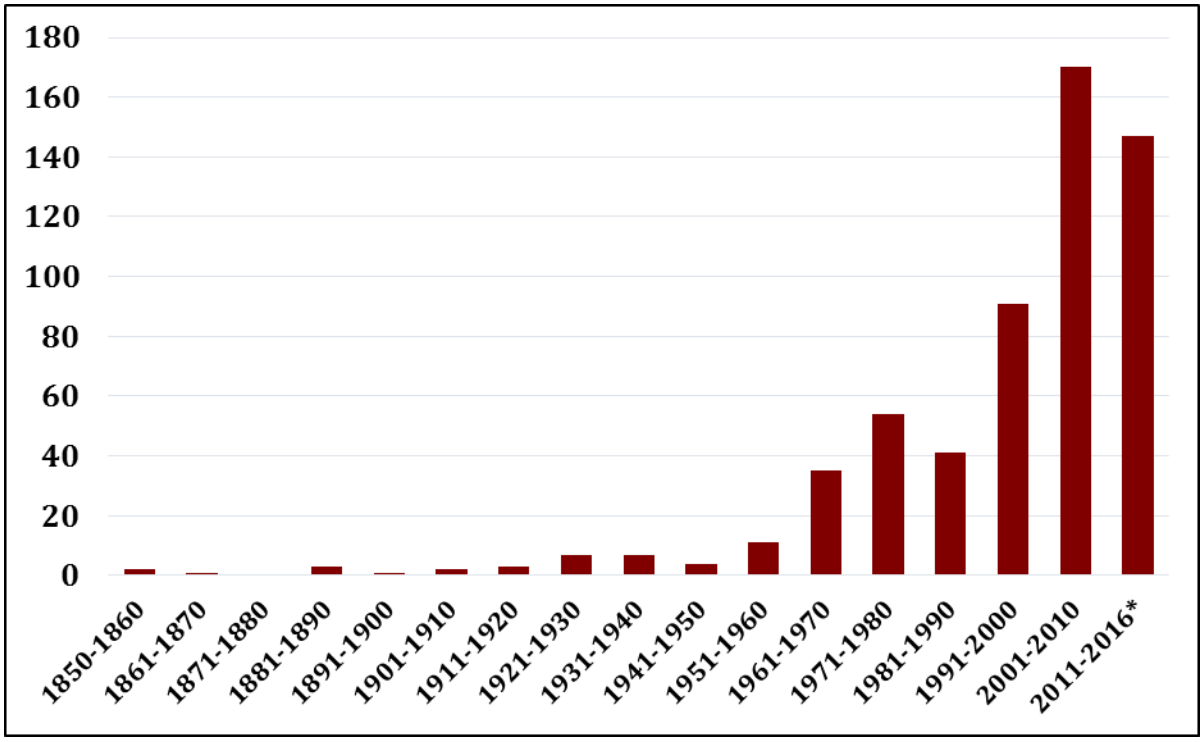

Figure 1.1. Number of publications examined related to microorganisms associated with Hemiptera by decade, 1850-2016. *Examined through June 2016.

Table 1.1. Symbiotic bacteria of Hemipteran insect families: association, acquisition, and publication reference.

Host Family	Symbiont	Association	Acquisition	Reference
Acanthosomatidae	*Rosenkranzia clausaccus*	Obligate (P)	Maternal (V)	Kikuchi 2009
Aleyrodidae	*Portiera aleyrodidarum*	Obligate (P)	Maternal (V)	Costa et al. 1993b
	Wolbachia spp.	Facultative (S)	Environmental (H)	Zchori-Fein and Brown 2002
	Arsenophonus spp.	Facultative (S)	Environmental (H)	Clark et al. 1992
	Rickettsia spp.	Facultative (S)	Environmental (H)	Gottlieb et al. 2006
	Hamiltonella spp.	Facultative (S)	Environmental (H)	Skaljac et al. 2010
	Cardinium spp.	Facultative (S)	Environmental (H)	Skaljac et al. 2010
	Fritschea spp.	Facultative (S)	Environmental (H)	Thao et al. 2003
	Bacillus spp.	Facultative (S)	Environmental (H)	Singh et al. 2012
	Enterobacter spp.	Facultative (S)	Environmental (H)	Singh et al. 2012
	Paracoccus spp.	Facultative (S)	Environmental (H)	Singh et al. 2012
	Acinetobacter spp.	Facultative (S)	Environmental (H)	Singh et al. 2012
Alydidae	*Burkholderia* spp.	Facultative (S)	Environmental (H)	Kikuchi et al. 2005
Anthocoridae	*Wolbachia* spp.	Facultative (S)	Environmental (H)	Zchori-Fein and Perlman 2004
Aphididae	*Buchnera aphidicola*	Obligate (P)	Maternal (V)	Buchner 1965
	Serratia symbiotica	Facultative (S)	Maternal (V) or Environmental (H)	Moran et al. 2005 Oliver et al. 2010
	Regiella insecticola	Facultative (S)	Maternal (V) or Environmental (H)	Moran et al. 2005 Oliver et al. 2010
	Hamiltonella defensa	Facultative (S)	Maternal (V) or Environmental (H)	Moran et al. 2005 Oliver et al. 2010
	Rickettsia spp.	Facultative (S)	Maternal (V) or Environmental (H)	Simon et al. 2007 Oliver et al. 2010
	Rickettsiella spp.	Facultative (S)	Unknown	Lukasik et al. 2013
	Erwinia aphidicola	Facultative (S)	Environmental (H)	Harada et al. 1997
	Staphylococcus xylosus	Facultative (S)	Environmental (H)	Fischer et al. 2015
Berytidae	*Burkholderia* spp.	Facultative (S)	Environmental (H)	Kikuchi et al. 2011a
Blissidae	*Burkholderia* spp.	Facultative (S)	Environmental (H)	Kikuchi et al. 2011a
Cicadellidae	*Baumannia cicadellinicola*	Obligate (P)	Maternal (V)	Moran et al. 2008
	Sulcia muelleri	Obligate (P)	Maternal (V)	Moran et al. 2008
	Arsenophonus spp.	Facultative (S)	Maternal (V)	Kobialka et al. 2015
	Wolbachia spp.	Facultative (S)	Unknown	Hail et al. 2011
	Delftia (formerly Pseudomonas) spp.	Facultative (S)	Unknown	Hail et al. 2011
	Pectobacterium spp.	Facultative (S)	Unknown	Hail et al. 2011
	Moraxella spp.	Facultative (S)	Unknown	Hail et al. 2011
	Serratia spp.	Facultative (S)	Unknown	Hail et al. 2011
	Bacillus spp.	Facultative (S)	Unknown	Hail et al. 2011
	Cardinium spp.	Unknown	Maternal (V)	Sacchi et al. 2008
	Asaia spp.	Unknown	Maternal (V)[b]	Marzorati et al. 2006

S-Secondary; P-Primary; V-Vertical; H-Horizontal

[a] via coprophagy (consuming bacteria-laden feces)

[b] proposed method of transmission

Table 1.1 continued

Cicadidae	*Sulcia muelleri*	Obligate (P)	Maternal (V)	Zhou et al. 2015
	"*Candidatus* Hodgkinia cicadicola "	Obligate (P)	Maternal (V)	Zhou et al. 2015
	Enterobacter spp.	Facultative (S)	Unknown	Zhou et al. 2015
	Pseudomonas spp.	Facultative (S)	Unknown	Zhou et al. 2015
	Pantoea spp.	Facultative (S)	Unknown	Zhou et al. 2015
	Streptococcus spp.	Facultative (S)	Unknown	Zhou et al. 2015
	Uruburuella spp.	Facultative (S)	Unknown	Zhou et al. 2015
Cimicidae	*Wolbachia* spp.	Obligate (P)	Maternal (V)	Hosokawa et al. 2010b
	Pseudomonas spp.	Facultative (S)	Environmental (H)	Meriweather et al. 2013
	Enterobacteria spp.	Unknown	Unknown	Hosokawa et al. 2010b
	Burkholderia spp.	Facultative (S)	Environmental (H)	Saenz et al. 2013
	Shigella spp.	Facultative (S)	Environmental (H)	Meriweather et al. 2013
	Sphingomonas spp.	Facultative (S)	Environmental (H)	Meriweather et al. 2013
	Methylobacterium spp.	Facultative (S)	Environmental (H)	Meriweather et al. 2013
Cixiidae	*Wolbachia* spp.	Unknown	Unknown	Bressan et al. 2009
	Sulcia muelleri	Unknown	Unknown	Bressan et al. 2009
	Arsenophonus sp.	Unknown	Environmental (H)[b]	Bressan et al. 2012
Coreidae	*Burkholderia* spp.	Facultative (S)	Maternal (V)	Kikuchi et al. 2011a
	Wolbachia spp.	Facultative (S)	Unknown	Olivier-Espejel 2011
Delphacidae	*Wolbachia* spp.	Facultative (S)	Environmental (H)	Subandiyah et al. 2000
Lygaeidae	*Wolbachia* spp.	Facultative (S)	Unknown	Kuechler et al. 2010
	Rickettsia spp.	Facultative (S)	Unknown	Kuechler et al. 2010
Miridae	*Rickettsia* spp.	Facultative (S)	Unknown	Caspi-Fluger et al. 2014
	Wolbachia spp.	Unknown	Maternal (V) and Environmental (H)[b]	Machtelinckx et al. 2012
Pachygronthidae	*Burkholderia* spp.	Facultative (S)	Environmental (H)	Kikuchi et al. 2011a
Pentatomidae	*Klebsiella pneumoniae*	Facultative (S)	Maternal (V)	Hirose et al. 2006
	Enterococcus faecalis	Facultative (S)	Maternal (V)	Hirose et al. 2006
	Erwinia spp.	Facultative (S)	Maternal (V)	Prado and Almeida 2009a
	Pantoea spp.	Facultative (S)	Maternal (V)	Prado and Almeida 2009a
	Spiroplasma spp.	Facultative (S)	Maternal (V)	Matsuura et al. 2014
	Sodalis spp.	Facultative (S)	Maternal (V)	Matsuura et al. 2014
	Rickettsia spp.	Facultative (S)	Maternal (V)	Matsuura et al. 2014
Plataspidae	*Ishikawaella capsulata*	Obligate (P)	Maternal (V)	Hosokawa et al. 2006
Pseudococcidae	*Tremblaya princeps*	Obligate (P)	Maternal (V)	Baumann et al. 2002
	Spiroplasma spp.	Facultative (S)	Environmental (H)	Fukatsu and Nikoh 2000
Psyllidae	*Carsonella ruddii*	Obligate (P)	Maternal (V)	Thao et al. 2000b
	Profftella armatura	Obligate (P)	Maternal (V)	Nakabachi et al. 2013
	Wolbachia spp.	Facultative (S)	Environmental (H)	Subandiyah et al. 2000
	Liberobacter spp.	Facultative (S)	Environmental (H)	Nachappa et al. 2011
	Gordonia spp.	Facultative (S)	Unknown	Hail et al. 2012
	Rhizobium spp.	Facultative (S)	Unknown	Hail et al. 2012
	Mycobacterium spp.	Facultative (S)	Unknown	Hail et al. 2012
	Xanthomonas spp.	Facultative (S)	Unknown	Hail et al. 2012
Pyrrhocoridae	*Coriobacterium glomerans*	Unknown	Maternal (V)	Kaltenpoth et al. 2009

Table 1.1 continued

Reduviidae	*Rhodococcus rhodnii*	Facultative (S)	Environmental (H)[a]	Eichler and Schaub 2002
	Arsenophonus triatominarum	Facultative (S)	Environmental (H)	Hypša and Dale 1997
	Serratia marcescens	Facultative (S)	Environmental (H)	Figueiredo et al. 1995
	Pseudomonas spp.	Facultative (S)	Environmental (H)	Figueiredo et al. 1995
Rhyparochromidae	*Burkholderia* spp.	Facultative (S)	Environmental (H)	Kikuchi et al. 2011a
Scutelleridae	*Sodalis*	Unknown	Maternal (V)	Kaiwa et al. 2010
Triozidae	*Carsonella ruddii*	Obligate (P)	Maternal (V)	Arp et al. 2014
	Wolbachia spp.	Unknown	Unknown	Arp et al. 2014

CHAPTER 2.

Growth Kinetics of Endosymbiont *Wolbachia* in the Common Bed Bug, *Cimex lectularius*

Abstract

The common bed bug, *Cimex lectularius* harbors the endosymbiotic microorganism *Wolbachia* (*w*Cle) in a gonad-associated bacteriome. *Wolbachia* infections provide an important obligate nutritional association with its host, but if *w*Cle is eliminated therapeutically, the bed bug experiences reduced reproductive fitness, and poor performance. The nature of endosymbiosis would suggest that all *C. lectularius* individuals in populations globally would harbor with *w*Cle. However, the early research and more recent studies have reported variation in both infection frequency and the relative abundance of *w*Cle in field-collected samples of bed bugs. While some lineages of bed bugs might have developed facultative associations with *Wolbachia*, these observations could also be a consequence of the intimate synchronization of their respective physiologies. The growth kinetics of *w*Cle in *C. lectularius* is poorly understood, but endosymbiont proliferation rates have been quantified in other organisms in relation to host development. The objective of this study was to quantify *w*Cle over the life cycle of two strains of *C. lectularius*. We extracted DNA from bed bugs and obtained absolute quantification of the 16S copy number of *Wolbachia* in 1st instars, 5th instars, and adults using a Droplet Digital PCR (ddPCR) system optimized for *Wolbachia* detection. Our results highlight that *w*Cle is dynamic during bed bug development, changing relative to life stage, intermolt stage, and blood-fed status. These results suggest new hypotheses about the coordination of *Wolbachia* growth and regression with its host's physiology and endocrine events. The observed quantitative modulation of *w*Cle during the bed bug life cycle and during periods of starvation may explain the disparities in *w*Cle infections reported in field-collected *C. lectularius*.

Introduction

Insect bodies are inhabited by diverse bacterial communities, ranging from commensal, parasitic to facultative or obligatory mutualistic associations with their host (Douglas 2015, Feldhaar 2011). The gut fauna often plays a role in nutrition, development, defense or communication, and it may consist of highly complex and somewhat varied communities of associated bacterial species (Douglas 2015, Engel and Moran 2013, Dillon and Dillon 2004). Gut microbiomes are generally horizontally transmitted and insects have evolved specialized strategies, such as coprophagy and proctodeal feeding, to acquire the proper microbes (Engel and Moran 2013). Several transovarially transmitted, obligate intracellular symbionts occur in insects that eat nutritionally poor or unbalanced diets; among these are *Blattabacterium* spp. in cockroaches, *Buchnera* in aphids, *Portiera* in whiteflies, *Baumannia* in leafhoppers, *Wigglesworthia* in tsetse flies, and *Wolbachia* in bed bugs. Interestingly, these bacteria are transmitted with high fidelity within lineages, so that all progeny of a carrier mother are infected. These "heritable" symbionts vary both in their contributions to their hosts and in their localization within the host, some residing systemically in various tissues, some localize diffusely in cells associated with the fat body or integument, while others inhabit a single cell type (mycetocyte) or specialized structures, bacteriomes (or mycetome), often associated with the gonads (Douglas 2015, Feldhaar 2011, Kikuchi 2009, Dale and Moran 2006).

The common bed bug, *Cimex lectularius* harbors a hereditary, Gram (-), intracellular α-proteobacterium *Wolbachia,* that is primarily concentrated in a gonad-associated bacteriome. *Wolbachia*'s relationship with *C. lectularius* presumably evolved from a

facultative association to obligate mutualism where the bacteria garner protection and nutrients within their host in exchange for supplementing the host's nutritional needs (Hosokawa et al. 2010, Nikoh et al. 2014, Moriyama et al. 2015). The *C. lectularius* strain of *Wolbachia* (*w*Cle) belongs in the F supergroup (Rasgon and Scott 2004), and its genome was found to be very similar to other insect-associated facultative *Wolbachia* strains in the A, B and D supergroups, but with the exception of the presence of genes encoding a complete biotin (vitamin B_7) and riboflavin (vitamin B_2) biosynthetic pathways (Nikoh et al. 2014, Moriyama et al. 2015). Bed bugs cured of *w*Cle with antibiotics lose fitness, exhibit poor adult emergence, and severely reduced egg hatch rates, but recover significantly with the addition of B vitamins (Hosokawa et al. 2010, Moriyama et al. 2015).

The obligate association of *w*Cle with its host, transovarial transmission, and poor performance of bed bugs cured of *w*Cle infection, all would suggest that all *C. lectularius* individuals in all populations should be infected with *w*Cle. Yet, early research (Arkwright et al. 1921, Hertig and Wolbach 1924) and more recent studies (Akhoundi et al. 2016, Fisher et al. unpublished data, Meriweather et al. 2013) reported variation in both infection frequency and the relative abundance of *w*Cle in field-collected samples of bed bugs. While it is possible that some *C. lectularius* lineages might have developed facultative associations with *Wolbachia*, these observations could also be a consequence of the intimate synchronization of their respective physiologies. For example, *w*Cle might experience proliferation-regression cycles in relation to the bed bug ingesting and processing a blood-meal, or in relation to bed bug molt and developmental stages.

The growth kinetics of *w*Cle in *C. lectularius* is poorly understood, but endosymbiont titers have been quantified in other organisms in relation to host development. For example, *Wolbachia* is dynamic in the nematode *Brugia malayi*, and it fluctuates over the course of the host's life cycle. The endosymbiont resides intracellularly inside host-derived vacuoles in the hypodermal cord cells (McGarry et al. 2004), and the number of *Wolbachia* per nematode cell nucleus remains low in second (L2) and third stage larvae (L3), but increases significantly with rapid multiplication in fourth stage larvae (L4), likely related to oogenesis (Fenn and Blaxter 2004, McGarry et al. 2004). The titer of the primary endosymbiont *Rhodococcus rhodnii* fluctuates over time in the gut of the blood-feeding insect relative of bed bugs, *Rhodnius prolixus*. Gut titers are highest ~5 days following ingestion of a bloodmeal, reaching as high as 10^8 colony-forming units ml^{-1} in the hindgut, but gradually decline over time (Dillon and Dillon 2004, Beard et al. 2002). Third, fourth, and fifth instar *R. prolixus* nymphs also have more *R. rhodnii* than 1^{st} or 2^{nd} instars (Baines 1956). Copy number of the *rrs* gene, that codes for 16S rRNA in the aphid endosymbiont *Buchnera*, increases in relation to aphid weight over the course of aphid development (Bauman and Baumann 1994), and *Buchnera* declines with host age (Komaki and Ishikawa 2000).

Nonetheless, to our knowledge, there are no reports that quantify the endosymbiont titer within the intermolt stage and in relation to feeding in bed bugs. We were particularly interested in this relationship as well as the *w*Cle titer before and after the nymphal and adult molts in *C. lectularius*. Moreover, we sought to understand whether the reported highly variable relative abundance of *w*Cle in field-collected *C. lectularius* could be attributed to variation in the bed bugs' developmental and blood-fed status. In this study, we quantified

the amount of *w*Cle during nymphal and adult development of *C. lectularius* using ddPCR, due to its increased accuracy of quantification at low target concentrations over qPCR (Gutiérrez-Aguirre et al. 2015, Dreo et al. 2014, Jones et al. 2014), and absolute quantification without the need for reference materials with known quantification or the generation of a standard curve (Gutiérrez-Aguirre et al. 2015, Dreo et al. 2014, Jones et al. 2014). The 16S rDNA gene of *Wolbachia* has been reported as a single copy in the two supergroup A strains *w*Mel and *w*Ri in *Drosophila melanogaster* and *Drosophila simulans* respectively (Klasson et al. 2009), in the supergroup B strain *w*Pip in the mosquito *Culex quinquefasciatus* (Klasson et al. 2008), and in the supergroup D strain *w*Bm in *Brugia malayi* (Foster et al. 2005). Similar to *Wolbachia* in A, B, and D supergroups, 16S rDNA copy number for *w*Cle exists as a single copy (Ribosomal Database Project, University of Michigan, https://rrndb.umms.med.umich.edu/). Our results suggest that the relative abundance of the *w*Cle endosymbiont fluctuates dramatically over the life cycle of *C. lectularius* and in relation to its blood-fed status. These results may also explain, at least in part, the high variation in infection frequency in field-collected bed bugs.

Materials and Methods

Insects. Two *Cimex lectularius* bed bug strains were used in these experiments. The Harold Harlan strain (HH,) was collected in 1973 in Ft. Dix, NJ, USA. It is an insecticide-susceptible strain used as a standard in many labs working on bed bugs. The same strain has been maintained at NC State University since 2008. The Jersey City (JC) strain of *C.*

lectularius was collected in 2008 in NJ and maintained at the NC State University since then. It is known to be highly resistant to pyrethroid insecticides (Barbarin et al. 2017, Romero and Anderson 2016). Both strains were reared at 27 ± 1°C (Thermo Scientific, Precision™ model#3727, Waltham, MA, USA) under a photocycle of 12/12 (light/dark). Bed bugs were maintained in small, round plastic containers that contained pleated paper shelters which contacted plankton netting on the top of the container through which bed bugs could feed on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA). Blood was placed in a water-jacketed custom-made glass feeder and warmed to 38°C with a thermal circulator.

Experimental design. Our objective was to determine if the relative abundance of *Wolbachia* fluctuated during the developmental cycle and in correlation with blood-fed status of *C. lectularius* for both HH and JC strains of the common bed bug.

Nymphal development. Neonate unfed 1st instars were placed into screen-capped 7 ml glass vials (6–12 nymphs per vial, 3 replicates) with pleated manila card stock strips for shelter. Bed bugs were fed defibrinated rabbit blood and maintained under the same conditions as described above for colony maintenance. To synchronize the manipulations for this experiment, one group of bed bugs were used as sentinels to predict molting events for the test group. These sentinel groups consisted of five 1st and five 5th instars held in separate vials. These were used to estimate the time interval between feeding and 1 d before the next molt. Bed bugs in this vial were fed the same batch of blood 3 days prior to commencing the experiment.

Three individuals from each vial (n =9) were randomly collected for testing at the following time intervals: unfed 1st instars, 1st instars 2 d post-feeding, and 1st instars 1 d before molting

to 2nd instar. Each bed bug was separately placed into a 1.5 ml microcentrifuge tube with 95% ethyl alcohol (EtOH). Samples were stored at -20°C until DNA extraction. The same procedure was repeated using newly molted 5th instar females, which were sampled as follows: unfed 5th instars, 5th instars 2 d post-feeding, and 5th instars 1 d before molting to adult.

Adult development. Sixty newly molted unfed 5th instar females were placed into a 7 ml screen-capped vial with pleated manila card stock, as above. These nymphs were fed to repletion and they were maintained in the same incubator as other nymphs and under the same conditions. Newly emerged unfed adults were placed in 7 ml screen-capped glass vials (12 females per vial, 3 replicates), as above, with pleated paper for shelter. Three unfed females from each vial (n =9) were collected before feeding, the rest of the adult females were fed defibrinated rabbit blood to repletion, and 3 individuals from each vial (n =9) were collected on the following days after feeding: 2, 10, 20, and 40 d. Collected females were placed individually into separate 1.5 ml microcentrifuge tubes in 95% EtOH and stored at -30°C until DNA extraction.

DNA extraction. Total genomic DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN, Germantown, MD, USA) using a modified purification of total DNA from animal tissues (spin-column) protocol. Heads were removed from 5th instars and adults, and individual bed bugs were placed in 1.5 ml microcentrifuge tubes with 180 µl of ATL buffer solution and homogenized using a sterile plastic pestle. Proteinase K (20 µl) and 4 µl of RNase was immediately added after homogenization, and samples were then digested overnight (~24 h) in a 56°C water bath. Samples were then vortexed for 15 s, 200 µl of AL

buffer was added, and then incubated in a 70°C water bath for 10 min. Following incubation, samples were vortexed for 15 s, and 200 µl of 96% EtOH was added. The mixture was then pipetted onto the spin column, and the DNA was bound. The columns washed with AW1 buffer and then washed twice with AW2 buffer to further remove salts. Finally, total DNA was eluted with 200 µl of AE buffer. All the DNA samples were stored at -30°C until quantification and further use.

Quantification of *Wolbachia.* To obtain absolute quantification of *Wolbachia* in each individual bed bug, we used a droplet digital PCR (ddPCR™) system (Model QX200, Bio-Rad Laboratories, Hercules, CA, USA). The *Wolbachia*-specific primers, adopted from Sakamoto and Rasgon (2006), targeted a region of the *Wolbachia* 16S rRNA gene that produced a 136 bp amplicon. We used primers for a ribosomal protein (RPL18) specific to *C. lectularius* as the reference gene due to its stability (Mamidala et al. 2011); it produced a 137 bp amplicon. We used double-quenched TaqMan probes with a 5' FAM fluorophore for *Wolbachia*, a 5' HEX fluorophore for *C. lectularius*, and 3' Iowa Black FQ quenchers with internal ZEN quenchers (Integrated DNA Technologies, Inc., Coralville, IA, USA) specific to each target. Primer and probe sequences are listed in Table 2.1. Template DNA was combined with *Wolbachia*-specific forward and reverse primers, TaqMan probes, and the ddPCR Supermix for Probes (Bio-Rad) into PCR-ready samples. The ddPCR reaction was optimized using extracted bed bug DNA that contained *Wolbachia* and bed bug DNA that contained no *Wolbachia* obtained from an established bed bug colony treated with antibiotics. Repeatability of the ddPCR was assessed for detection of *Wolbachia*-free DNA and bed bug DNA on five different days and different experiments, and mean copy number

(± SE) were used to calculate the coefficient of variation. Conventional PCR was used to verify amplification and respective band size with a 25 µl reaction of 12.5 µl GoTaq® Green 2x Master Mix (Promega, Madison, WI, USA), 2.5 µl of template DNA, and 2.5 µl of each primer set (Table 2.1) under the following protocol: 95°C for 2 min and (95°C for 30 s, 60°C for 30 s, 72°C for 1 min) x 36 cycles, and 72°C for 5 min.

The bed bug/*Wolbachia* ddPCR assay comprised 22 µl of 1 x Droplet Supermix (Bio-Rad), 5 µl of genomic DNA isolated from a bed bug, 2 U of *Mse*I restriction enzyme (New England Biolabs, Ipswich, MA), 500 nM each of forward and reverse primers and 250 nM each of FAM- or HEX-labeled TaqMan probes for bed bug and *Wolbachia* strains, respectively. The 22 µl of PCR mixtures were partitioned into an emulsion of ~20,000 droplets using the ddPCR system. PCR was performed on a T100 Thermal Cycler using the following protocol: 95°C for 10 min and (94°C for 30 s, 56°C for 2 min) x 40 cycles, and 98°C for 10 min. Post PCR, droplets were analyzed on the QX200 Droplet Reader. Absolute DNA copy numbers of bed bug and *Wolbachia* sequences in a sample were calculated on the Poisson distribution using the Quantasoft software version 1.7.4 (Bio-Rad). Data are reported as *Wolbachia* DNA (16S rRNA) copy number per individual bed bug and as *Wolbachia* DNA copy number per *C. lectularius* DNA (RPL18) copy number. Bed bug DNA samples containing *Wolbachia* (+ control) and without *Wolbachia* (- control) were included in each experiment as checks on the ddPCR results. A no-template control was also included in each experiment to control for non-specific amplifications. To estimate the quantification capacity of the ddPCR assay, serial dilutions of a DNA sample were prepared in water (5-, 25-, 125-, 625-, 3125-, and 15625-fold dilutions).

Quantitative analysis of differences in *Wolbachia* abundance. We generated descriptive statistics in SPSS Version 19 (IBM Corp., Armonk, NY, USA). We also used a General Linear Model (GLM) Univariate Analysis of Variance (ANOVA) and Tukey's HSD ($\alpha = 0.05$) in SPSS to estimate significant differences in *Wolbachia* copy number (mean ± SE) per bed bug within each life stage and across all life stages. Samples with low DNA yield (<15 copies/µl of the bed bug reference gene RPL18 or *Wolbachia* 16S target) were not included in the analysis.

Results

We designed a ddPCR duplex assay for the absolute quantification of target gene copy number in bed bugs and their associated *Wolbachia*. The ddPCR optimization resulted in little variation in samples, where coefficients of variation of estimated DNA copy numbers of bed bug and *Wolbachia* in control samples were 2.1% and 2.4% respectively, with a mean (± SE) copy number of bed bug DNA (RPL18) of 632,480 ± 3565 per bed bug, and 385,920 ± 4473 of *Wolbachia* DNA (16S rRNA) per bed bug (Fig. 2.1A). The *Wolbachia*-free control bed bugs removed from antibiotics after 90 d contained no copies of *Wolbachia* (Fig. 2.1B), a mean (± SE) copy number of RPL18 of 532,480 ± 7634, and no DNA was detected in the no-template control (Fig. 2.1C). However, 1 HH bed bug and 5 JC bed bugs were excluded from further analysis because they contained <15 copies/µl of the bed bug RPL18 or *Wolbachia* 16S rRNA genes.

Mean (± SE) *Wolbachia* copy number per bed bug ranged from 45,428 ± 15,349 in 1st instars 1 d before the molt to 2nd instar to 2,063,796 ± 484,523 in newly emerged unfed adult

females in the HH strain, and from 15,865 ± 3615 in unfed 1st instars to 2,672,853 ± 551,627 in newly emerged unfed adults in the JC strain. Adults and late 5th (last) instars had the greatest amount of variation in *Wolbachia* copy number per bed bug (Table 2.2). Significant differences in *Wolbachia* copy number per bed bug were observed between all groups (1st instars, 5th instars, adult females) in both HH (F = 2.99; df = 20, 77; P = 0.0003) and JC (F = 4.97; df = 20, 73; P < 0.0001) bed bug strains.

First instars.

Wolbachia copy number per bed bug in the HH strain remained low ($\bar{X}$ = 60,449) and was not significantly different among the three stages of 1st instars (F = 0.86; df = 2, 23; P = 0.4370). The *Wolbachia* titer remained steady per 1st instar bed bug 2 d after feeding, but declined just before the molt to 2nd instar (Fig. 2.2A). In contrast, in the JC strain *Wolbachia* copy number per bed bug significantly increased 3-fold from the start to the end of the 1st instar (F = 4.95; df = 2, 21; P = 0.0173). This pattern is best illustrated in Fig. 2.3B, where *Wolbachia* titer is normalized relative to the respective titer in unfed 1st instars. However, the ratio of *Wolbachia* copy number to bed bug DNA copy number, which normalizes for bed bug stage and size, increased monotonically in both strains during the 1st instar (Fig. 2.2B).

Fifth instars.

In both bed bug strains there was a large increase in *Wolbachia* copy number per bed bug between the end of the 1st instar and the start of the 5th instar, ~22-fold increase in HH bugs and ~15-fold increase in JC bugs (Fig. 2.2A). The three stages of HH 5th instars did not differ significantly (F = 0.5490; df = 2, 24; P = 0.5846), and neither did the JC 5th instars (F = 0.60; df = 2, 24; P = 0.5586). In both strains a slight decline (~42% in HH, ~37% in JC) in

blood meal-fed bugs was followed by a slight increase in *Wolbachia* copy number per bed bug just before the adult molt. The ratio of *Wolbachia* copy number to bed bug DNA copy number continued to increase through the end of the 5th instar, indicating 1.3–1.9-fold faster proliferation of *Wolbachia* DNA than host DNA during this period in both bed bug strains.

Adult females.

Another large increase in *Wolbachia* copy number per bed bug occurred within one day between the last instar and the adult molt (~2-fold increase in HH bugs, ~3.3-fold increase in JC bugs). Subsequently, in the adult stage *Wolbachia* copy number per bed bug declined dramatically with prolonged starvation. Significant differences were observed in *Wolbachia* copy number per bed bug among starved adults in both the HH strain (F = 11.18; df = 4, 40; $P < 0.0001$) and JC strain (F = 10.47; df = 4, 38; $P < 0.0001$). *Wolbachia* copy number per HH bed bug declined by 95.5% 40 d after feeding, and in JC this decline was ~93.0% (Fig. 2.2A).

We generated two normalizations to observe the patterns of *Wolbachia* copy numbers in both strains. In the first normalization, the respective *Wolbachia* DNA copy number per unfed 1st instar was set to unity and all subsequent values were normalized relative to it (Fig. 2.3A). Dramatic differences were noted between the two strains. Whereas *Wolbachia* DNA copy number per bed bug in the HH strain increased 15- and 30-fold in unfed 5th instars and adults, respectively, in the JC strain these increases were 44- and 168-fold. Nevertheless, the overall patterns in both strains were similar.

A second normalization, within each life stage, was relative to unfed 5th instars and unfed adult females (Fig. 2.3B). As already mentioned, the patterns in 1st instars were remarkably

different between the two strains. The patterns for 5th instars and adults were similar in both strains when the absolute *Wolbachia* DNA copy number per bed bug was normalized to the beginning of each developmental stage.

Discussion

Our results highlight that *Wolbachia* in the bed bug (*w*Cle) is dynamic during bed bug development, changing relative to life stage, intermolt stage, and blood-fed status. Overall, we observed that (1) Neonate unfed, lab-reared, bed bugs (HH strain) had 4.3-fold more *w*Cle per bug than field-collected bugs (JC strain), and when normalized to the amount of bed bug DNA a 1.7-fold difference remained; (2) *w*Cle 16S copy number per bed bug increased ~30-fold (HH) and ~168-fold (JC) between the 1st instar and adult stages; (3) The largest increase (~15-fold HH, ~44-fold JC) was between the 1st and 5th instars; (4) *w*Cle DNA copy number increased throughout both 1st and 5th instars relative to bed bug DNA copy number; (5) In both 5th instars and adult females, *w*Cle levels declined significantly within 2 days of ingesting a blood-meal; (6) In adults, *w*Cle decreased by ~95% (HH) and ~93% (JC) after 40 d of starvation to amounts approaching those observed in 1st instars; and (7) Adults of the JC field-collected strain accumulated more *w*Cle per bug and also relative to amounts of bed bug DNA than the lab-maintained HH strain, and they retained more *w*Cle through starvation. Moreover, we conclude that ddPCR is highly sensitive and therefore appropriate for quantifying absolute amounts of *Wolbachia* in bed bugs as well as *Wolbachia* DNA relative to bed bug DNA. Finally, the observed modulation of the amount of *w*Cle

during the bed bug life cycle and during periods of starvation may explain the disparities in *w*Cle infections reported in field-collected *C. lectularius*.

Endosymbiotic bacteria, such as *Wolbachia*, are broadly associated with many insect species in a variety of parasitic, commensal and mutualistic associations (Douglas 2015, Feldhaar 2011). As diverse is *Wolbachia*'s location within the host, ranging from a broad systemic distribution, to specific tissues such as fat body cells, or specialized and highly localized bacteriomes (Douglas 2015, Feldhaar 2011, Kikuchi 2009, Dale and Moran 2006). In parasitic associations, particularly those involving reproductive manipulation of the host, we expected *Wolbachia* to be systemically distributed, in relatively low abundance, and its titer in the host to spike during the reproductive stage, in preparation for *Wolbachia*'s transovarial transmission. In contrast, for obligate mutualisms, *Wolbachia* is required for proper growth and development and is therefore expected to be localized in specific tissues and maintain relatively high populations that provision nutrients to all life stages of its host (Zug and Hammerstein 2015, Nikoh et al. 2014, Hosokawa et al. 2010).

A central feature of the association of *Wolbachia* with *C. lectularius* is their obligate mutualism, where *w*Cle provisions the bed bug with B vitamins in exchange for being hosted and transmitted as a "hereditary" component of the oocyte. *w*Cle are therefore essential for nutrient synthesis and embryonic development. *w*Cle-cured bed bugs grow more slowly and experience lower fecundity, but these effects can be reversed with biotin (vitamin B7) (Hosokawa et al. 2010, Nikoh et al. 2014) and riboflavin (vitamin B2) (Moriyama et al. 2015) supplementation of normal blood. This is similar to *Wolbachia* in supergroups C and D, which are associated with filarial nematodes, do not manipulate reproduction of the host,

and have obligatory associations with their host, and where fitness of nematodes is compromised when the *Wolbachia*-association is disrupted (Fenn and Blaxter 2004, McGarry et al. 2004). Interestingly, *w*Cle belongs to supergroup F, which contains *Wolbachia* strains that associate with both insects and filarial nematodes (Rasgon and Scott 2004). It is likely that because of *w*Cle's nutritional contribution to the bed bug, its titer increases in relation to bed bug growth and development, as we observed. The *w*Cle intimate association of the bacteriome with the gonads likely drives *w*Cle population dynamics.

The blood-meal is the only external source of nutrients for bed bugs, and indirectly for *w*Cle. In contrast to other hematophagous insects such as adult fleas or lice that reside exclusively on their host, bed bugs take larger infrequent blood meals and substantial degradation of erythrocytes is delayed up to 12 hours (Vaughn and Azad 1993). Nearly 50% of the blood meal (water weight) is excreted in fecal spots in the first 5 hours post ingestion (Omori 1941). Within just 2 days after 5^{th} instar and adult bed bugs ingest blood, the amount of *w*Cle decreases relative to the respective unfed stage (Fig. 2.2A). It is important to note, however, that relative to the amount of bed bug DNA, *w*Cle continues to increase throughout the 5^{th} instar (Fig. 2.2B). Whereas the pattern of *w*Cle per 5^{th} instar bug could suggest that *w*Cle might be responding to diminishing energy resources after the blood-meal is digested, two lines of evidence argue to the contrary. First, *w*Cle rebounds late in the same instar in the absence of feeding, and second, *w*Cle copy number increases throughout the 5^{th} instar in relation to *Cimex* DNA copy number. This pattern suggests fine coordination between the physiology of the bed bug and *w*Cle; when blood is available both host and symbiont grow.

The combined effects of starvation and bed bug reproduction also affect *w*Cle populations. As in 5th instars, the ratio of *w*Cle DNA to bed bug DNA increased after the blood-meal, but in nymphs, *w*Cle continued to decrease with prolonged starvation to extremely low levels. Although these females were unmated, *C. lectularius* virgin females oviposit some infertile eggs (Johnson 1941) and *w*Cle was provisioned to oocytes and lost with oviposited eggs. In the absence of re-feeding, the *w*Cle titer continued to decline. We predict that if females were offered a blood-meal >5 d after their last feeding, and mated to stimulate oocyte development and greater oviposition, *w*Cle numbers would dramatically increase. But this speculation would need to be tested empirically.

The fine coordination of *w*Cle titer with bed bug feeding needs further investigation. *w*Cle appears to respond to nutritional conditions in its host much faster than the time resolution of our study. It is possible, for example, that we missed peaks in *w*Cle populations immediately after the blood-meal that damped out within 2 d after the blood-meal was ingested. Highlighting this response is *w*Cle's rapid increase (2-fold in HH, 3.3-fold in JC) in one day between the late 5th instar and the newly emerged adult (Fig. 2.2A). Notably, since bed bug DNA increased during the molt, the ratio of *w*Cle to bed bug DNA in fact declined in the transition from nymph to adult (Fig. 2.2B).

Precise mechanisms of coordination of insect-bacterial symbiosis are not well known. Buchner (1965) concluded that in each host-symbiont example he examined, the host was the ultimate regulator of the symbiosis; binary fission occurred at much lower rates *in vivo* compared to related free-living bacteria (*in vitro*). In aphids and leafhoppers, lysosomal activity within mycetocytes selectively breaks down certain symbionts (Houk and Griffiths

1980, Griffiths and Beck 1973), and Hinde (1971) believed that in aphids, the selectivity removed nonviable individuals or was the primary means of insoluble nutrient acquisition from symbionts. Chang and Musgrave (1973) also discovered lysis of symbionts via autophagic vacuoles within the mycetome they termed 'cytolysomes' in *C. lectularius*, which suggested a host mechanism of symbiont suppression.

One could postulate that *w*Cle has relinquished control over replication and cell division to its bed bug host through lateral gene transfer or genome reduction, since this symbiosis is a highly-specialized obligate mutualism. Interestingly, this does not appear the case. In contrast to other *Wolbachia* genomes, *w*Cle has not undergone extensive genome reduction, or experienced significant loss of genes that control cell division, replication, or are responsible for other essential functions (Nikoh et al. 2014).

The coordination of *w*Cle with host development and physiology may be driven by nutrition or the bed bug's endocrine cycle. *Wolbachia*, like other intracellular endosymbionts such as *Spiroplasma*, require macronutrients from the host for replication and proliferation (Herren et al. 2014, Sinkins 2013, Chang 1984). Starvation reduced and eliminated *Wolbachia* in the predatory mite *Metaseiulus occidentalis* (Wu and Hoy 2012). Dietary intake strongly influences *Wolbachia* titer in the female *Drosophila* germline; a diet high in sucrose increases *Wolbachia* oocyte titer, but a high yeast diet decreases *Wolbachia* titer in oocytes (Serbus et al. 2015). Additionally, the ratio of protein to carbohydrate intake modulates *Wolbachia* abundance in *Drosophila* (Ponton et al. 2015). Glucose metabolism and glycogen storage in *B. malayi* are linked with *Wolbachia* fitness in a metabolic co-dependency pathway shared between the bacteria and its nematode host (Voronin et al.

2016). In the *B. malayi* system (*w*Bm) lacked genes for 2 glycolytic enzymes, 6-phosphofructokinase and pyruvate kinase, and were unable to convert glucose into pyruvate (Voronin et al. 2016).

Competition between *Wolbachia* infections and host nutrients has also recently been suggested. *Wolbachia* had a direct impact on cholesterol availability in *Aedes aegypti* mosquitoes (Caragata et al. 2014); *Wolbachia*-infected mosquitoes had ~25% less cholesterol than uninfected. Fallon et al. (2014) reported that depletion of host cell riboflavin reduced *Wolbachia* infection in cultured mosquito cells, suggesting that *Wolbachia* responded to the availability of riboflavin. In the case of *w*Cle, the bed bug host may out-compete *w*Cle for carbohydrates, lipids, or proteins, and hence offer an explanation to the substantial decline in *w*Cle we observed after bloodmeal ingestion.

In the related hemipteran *Rhodnius prolixus*, a blood-meal stimulates a molt cycle through humoral factors and neuronal signals generated by stretch receptors in the gut (Adams 1999). Molting in most insects is initiated by the corpora cardiaca release of prothoracicotropic hormone, which stimulates the prothoracic gland to produce ecdysone. In concert with a low juvenile hormone titer in last instars, and adult molt ensues. Ecdysone is a strong candidate for coordinating host and symbiont physiology. For example, in other relationships it promotes growth, maturation and sexual differentiation in flagellate protozoans that reside in the hindgut of the wood-feeding cockroach *Cryptocercus*. Thus, when ecdysone production was suppressed in the cockroach, gametogenesis of the protozoan *Trichonympha* ceased within 2 hours, and death occurred after 6-10 hours (Cleveland 1959). Conversely, ecdysone injections stimulated *Trichonympha* gametogenesis in cockroach

adults and nymphs (Cleveland et al. 1960). Similarly, juvenile hormone affected intracellular symbionts in the cockroach *Periplaneta americana* (Liu 1974). Bacterial symbionts in fat body mycetocytes of four species of cockroaches responded to changes in cockroach life stage and oocyte development and were affected by hormones from the corpora cardiaca (Milburn 1966). In bed bugs too, *w*Cle may be responding to fluctuations in hormone titers, and proliferate just before the molt.

The results presented in this study suggest new hypotheses about the coordination of *Wolbachia* growth and regression with its host's physiology and endocrine events. Future experiments could include quantitative measurements of *Wolbachia*'s rapid response to feeding, molting, mating, and oviposition, as well as to manipulations of juvenile hormone and ecdysone titers in the bed bug. As well, the broad-scale changes in *w*Cle in various life stages of *C. lectularius* bear on recent failures to detect *w*Cle in some field-collected bed bugs. This appears to be in conflict with the ostensible obligate symbiosis of *w*Cle and *C. lectularius*, suggesting (1) that *w*Cle titers in some field-collected bugs were below the detection limit of the assays, or (2) that *C. lectularius* lineages may vary in their degree of dependence on *w*Cle. Regarding the latter, the field-collected JC strain had much higher titers of *w*Cle than the lab-adapted HH strain, suggesting that the lab bed bugs might have adapted to frequent blood-meals by depending less on *w*Cle. Conversely, multiple recent surveys indicate that sizeable human populations in developed countries fail to consume the minimum recommended quantity of B-vitamins (Kennedy 2016); bed bugs feeding on B deficient people might depend more on their *Wolbachia* associates to provision them with B-

vitamins. Importantly, the *w*Cle-*Cimex* nutritional symbiosis has yet to be investigated with natural field populations.

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Table 2.1. *Wolbachia*-specific (*w*Cle) and *Cimex lectularius* (*Clec*) reference gene primer set and TaqMan probe sequences used in PCR and ddPCR assays.

Primer/Probe	Sequence (5'-3')	Reference
INTF2	AGTCATCATGGCCTTTATGGA	Sakamoto and Rasgon 2006
INTR2	TCATGTACTCGAGTTGCAGAGT	Sakamoto and Rasgon 2006
*w*Cle Probe	TGGTGTCTACAATGGGCTGCAAGG	This study
RPL18F	GTATGACGGAGGCAGCTAGG	Mamidala et al. 2011
RPL18R	AACATTCGAGCAAATTCGGTA	Mamidala et al. 2011
Clec Probe	ATGAGGACGGTGTTCTTGCCTGTC	This study

Table 2.2. Mean copy number (± SE) of *Wolbachia* 16S rRNA gene per bed bug in Harold Harlan and Jersey City bed bug strains. *N* = 9, except where indicated. Copy number is normalized in parenthesis relative to unfed individuals of the same life stage (= 1).

	Wolbachia 16S rRNA gene copy number per bed bug	
Group	Harold Harlan (HH)	Jersey City (JC)
1st instar: unfed	68,420 ± 19,358[a] (1)	15,865 ± 3615[b] (1)
1st instar: 2 d after feeding	68,386 ± 7138 (1.00)	40,205 ± 7464[a] (2.53)
1st instar: 1 d before molt	45,428 ± 15,349 (0.66)	47,540 ± 8488[a] (3.00)
5th instar: unfed	1,020,702 ± 180,361 (1)	695,396 ± 272,726 (1)
5th instar: 2 d after feeding	594,391 ± 110,048 (0.58)	434,818 ± 93,401 (0.63)
5th instar: 1 d before molt	1,002,809 ± 522,575 (0.98)	802,169 ± 310,564 (1.15)
Adult[c]: unfed	2,063,796 ± 484,523 (1)	2,672,853 ± 551,627 (1)
Adult[c]: 2 d after feeding	619,129 ± 137,433 (0.30)	1,915,173 ± 366,111 (0.72)
Adult[c]: 10 d after feeding	450,466 ± 116,461 (0.22)	1,132,951 ± 106,656 (0.42)
Adult[c]: 20 d after feeding	340,853 ± 60,254 (0.17)	395,824 ± 130,008[a] (0.15)
Adult[c]: 40 d after feeding	93,524 ± 17,617 (0.05)	187,044 ± 75,062[a] (0.07)

a = one replicate excluded
b = two replicates excluded
c = females only

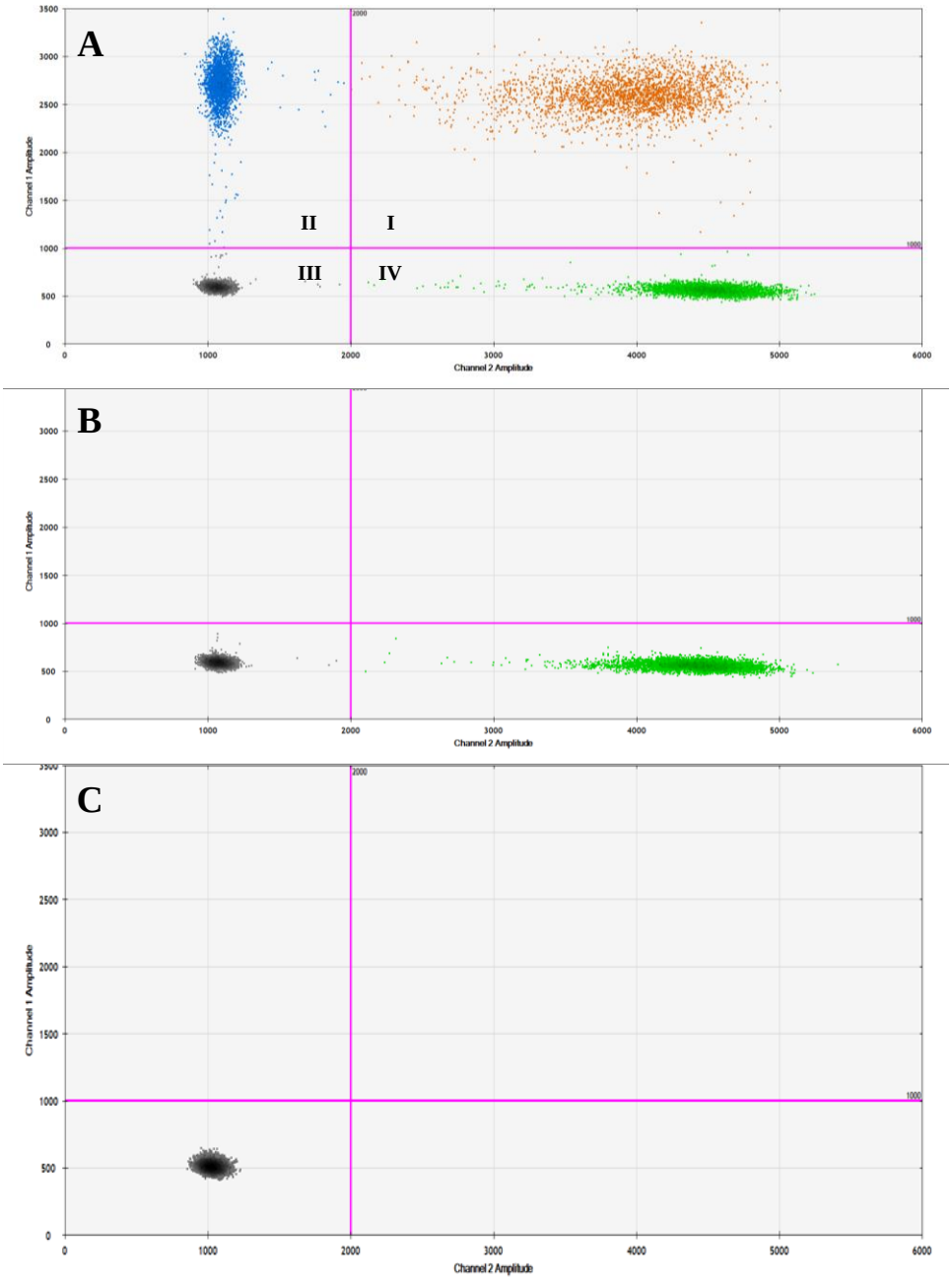

Figure 2.1. Droplet digital PCR optimization results. Copy number/µl of DNA for *Wolbachia* and *Cimex lectularius* (A), *Wolbachia*-free *Cimex lectularius* removed from antibiotics 90 d maintained on vitamins only (B), and no-template control (C). *Wolbachia* droplet spectrum (blue) in quadrant II, *Cimex lectularius* droplet spectrum (green) in quadrant IV, droplets with both targets (orange) in quadrant I, and droplets with neither target (gray) in quadrant III.

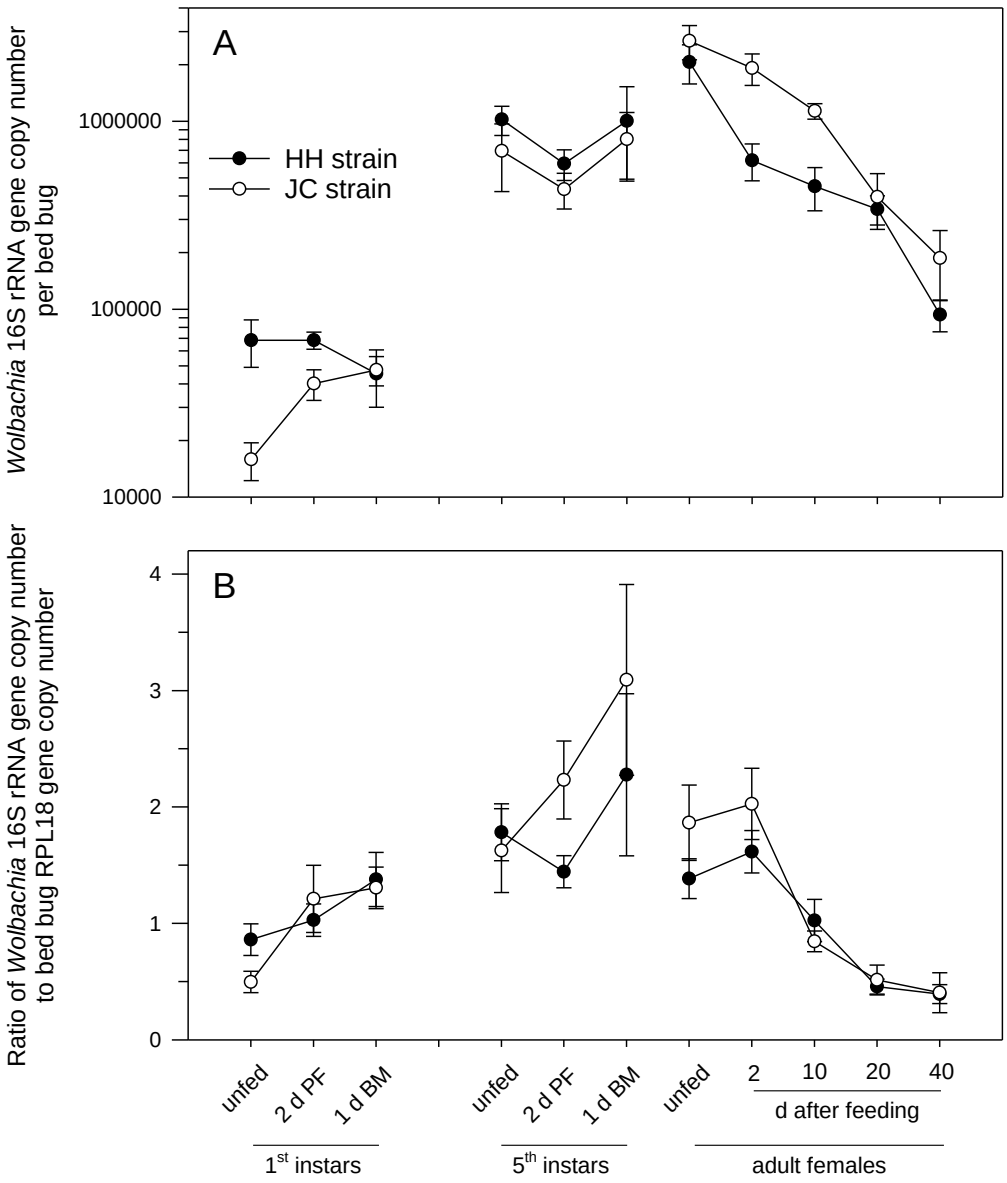

Figure 2.2. Mean (± SE) copy number of *Wolbachia* 16S rRNA gene per bed bug across development of the Harold Harlan (HH) and Jersey City (JC) strains of *Cimex lectularius* (A, note logarithmic scale) and the ratio the of *Wolbachia* 16S copy number to RPL18 reference gene copy number per bed bug (B). For each nymphal stage (1^{st} and 5^{th}), represented are unfed newly molted nymphs, 2 days post-feeding (2 d PF), and 1 day before the molt to the next stage (1 d BM). For adult females days after a blood-meal are shown.

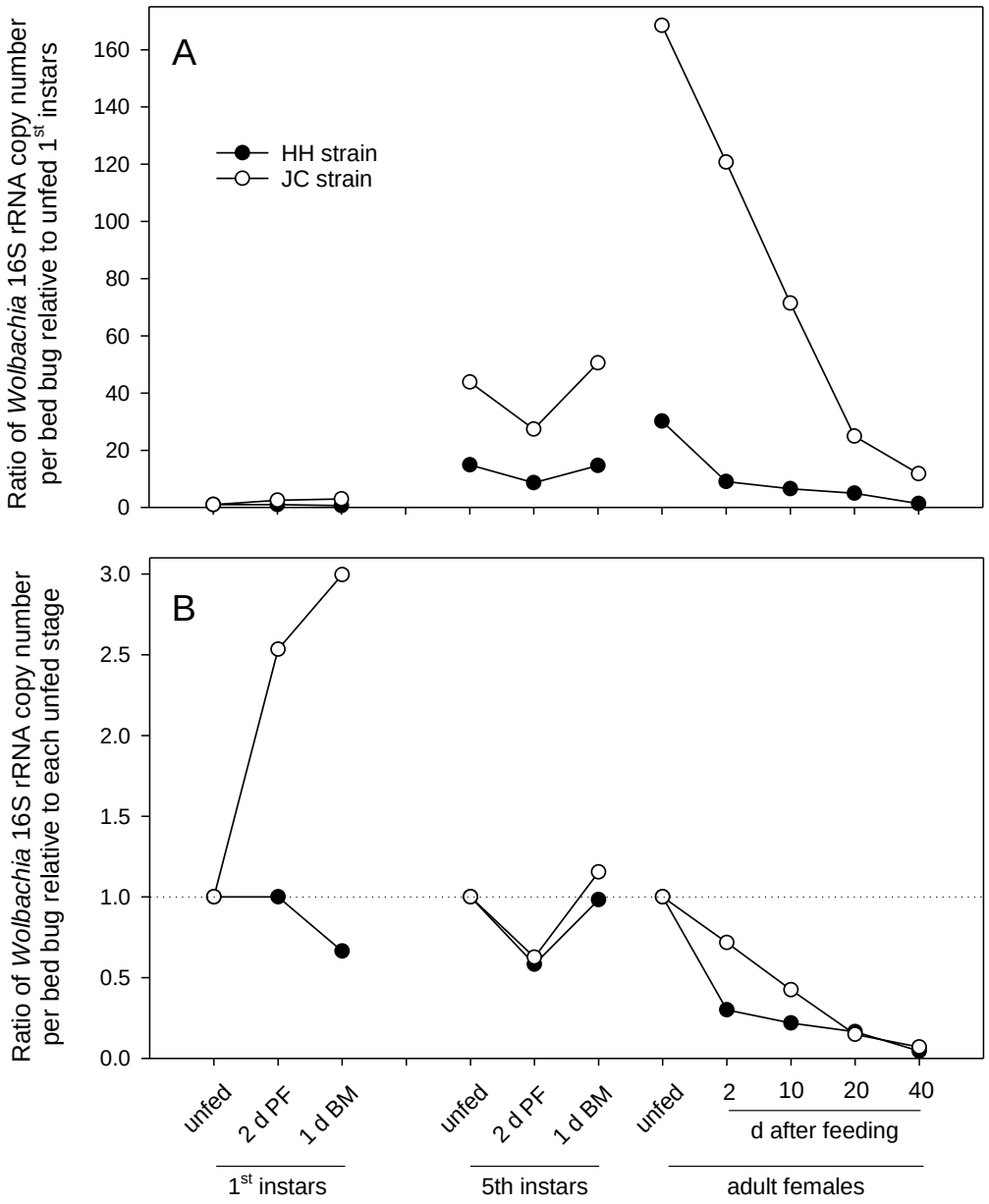

Figure 2.3. The ratio of copy number of *Wolbachia* 16S rRNA gene per bed bug across development relative to unfed 1st instars (A) and relative to unfed bed bugs of each of the three life stages (B) for both the Harold Harlan (HH) and Jersey City (JC) strains of *Cimex lectularius*. For each nymphal stage (1st and 5th), represented are unfed newly molted nymphs, 2 days post-feeding (2 d PF), and 1 day before the molt to the next stage (1 d BM). For adult females days after a blood-meal are shown.

CHAPTER 3.

Horizontal Transmission of *Wolbachia* in the Common Bed Bug, *Cimex lectularius*

Abstract

Symbiotic microorganisms are found throughout arthropod species. Primary and secondary symbionts are transmitted vertically from mother to offspring, but secondary symbionts are frequently acquired by novel hosts through horizontal transmission (HT). *Wolbachia* exists as both a primary and a secondary symbiont in insects, capable of colonizing hosts by both vertical transmission (VT) and HT. Mutualistic associations of *Wolbachia* within insects are maintained throughout natural populations almost exclusively through VT, including the obligate mutualism system in the bed bug *Cimex lectularius*. In some hematophagous insects, HT of facultative *Wolbachia* occurs mainly via sexual transfer from an infected male to uninfected female. Horizontal transmission of *Wolbachia* in bed bugs is poorly understood. The aim of this study was to investigate the possibility of sexual HT of *Wolbachia* between mating adults and to the progeny of *Wolbachia*-free females that mated with normal males. We used an established line of *Wolbachia*-free bed bugs to set up assays to examine transfer of *Wolbachia* from female to male, male to female, and female to male to female. Quantification of *Wolbachia* was performed with droplet digital PCR that was highly sensitive to both bacterial and bed bug DNA even at low concentrations. Our results suggest that *Wolbachia* could not re-establish in *Wolbachia*-free adults after copulation with *Wolbachia*-infected mates. At the same time however, *Wolbachia*-infected males appeared capable of transferring the endosymbiont to their progeny, either through the fertilized eggs of the *Wolbachia*-free mothers or through subsequent contact of the nymphs with the male or his feces. Our results invite new hypotheses to explore the bed bug-*Wolbachia* evolutionary relationship further, including research into coprophagous behaviors in Cimicidae.

Introduction

Symbiotic microorganisms, both primary and secondary, are widespread among arthropods. Primary (obligate) symbionts are those that have coevolved from ancient associations, whereas secondary (facultative) symbionts are acquired by and colonize naïve hosts (Dale and Moran 2006). Obligate symbionts are exclusively transmitted vertically, while facultative symbionts, although also transmitted vertically, are frequently transmitted horizontally via either sexual or environmental means (Douglas 2015, Ebert 2013). Primary symbionts typically increase host fitness, but secondary symbionts in a novel host can be either beneficial or deleterious to the host (Dale and Moran 2006, Werren et al. 2008, Werren and O'Neill 1997). While long-term viability of symbionts requires host to host transmission, horizontal transmission (HT) and vertical transmission (VT) are not mutually exclusive, and several symbionts, including *Wolbachia*, exhibit both modes referred to as mixed mode transmission (MMT) (Ebert 2013). Successful transmission from host to host depends on environmental factors, as well as host density, reproductive rate, mating frequency, and expected survival rate (Ebert 2013). Overall, HT of symbionts tends to favor parasitism, while VT of symbionts evolves toward mutualism (Zug and Hammerstein 2015).

The Gram (-) α-proteobacterium *Wolbachia* is estimated to exist in 40-66% of terrestrial arthropod species (Zug and Hammerstein 2012, Hilgenboecker et al. 2008). *Wolbachia* occurs in at least 1 million insect species (Werren et al. 2008) through a variety of parasitic, commensal, and mutualistic associations (Douglas 2015, Zug and Hammerstein 2015, Gill et al. 2014, Feldhaar 2011), occupying different locations within the host that range from widespread systemic distributions, to specific tissues such as fat body cells, or

specialized and highly localized bacteriomes (Douglas 2015, Feldhaar 2011, Kikuchi 2009, Dale and Moran 2006). Mutualistic associations of *Wolbachia* within insects are maintained throughout natural populations almost exclusively through VT from mother to developing offspring, but the global distribution of facultative *Wolbachia* in novel invertebrate hosts is attributed to HT (Werren et al. 2008, Raychoudhury et al. 2009). The broad phylogenetic evidence for recurrent HT of *Wolbachia* in arthropods provides evidence why mutualism with *Wolbachia* has not evolved more frequently (Zug and Hammerstein 2015). Nevertheless, *Wolbachia* should be classified as a MMT symbiont, but its mode of transmission varies among its many hosts (Ebert 2013, Moran et al. 2008).

The *Cimex lectularius* strain of *Wolbachia* (*w*Cle) belongs in the F supergroup and its genome was found to be similar to other insect-associated facultative *Wolbachia* strains in the A, B and D supergroups (Rasgon and Scott 2004). As one of the few examples of obligate mutualism with *Wolbachia*, *w*Cle contains genes encoding complete biotin (vitamin B_7) and riboflavin (vitamin B_2) biosynthetic pathways (Nikoh et al. 2014, Moriyama et al. 2015), and it provides B vitamins to its host in exchange for being harbored by the bed bug (Hosokawa et al. 2010, Moriyama et al. 2015). This relationship that presumably evolved from a once facultative association (Nikoh et al. 2014) is essential for embryonic development, growth and reproduction because bed bugs "cured" of *w*Cle with antibiotics exhibit poor adult emergence and severely reduced egg hatch. However, they recover significantly with supplementation of B vitamins in their blood meals (Hosokawa et al. 2010, Moriyama et al. 2015). As an obligatory endosymbiont, *w*Cle is vertically transmitted as a "heritable" component of the oocyte.

Although the main route of *Wolbachia* transmission occurs via vertical maternal deposition into developing oocytes, horizontally-acquired facultative transmission of *Wolbachia* has been documented in arthropods (Sicard et al. 2014, Dale and Moran 2006). HT occurs by way of co-feeding on the same plant in many plant-feeding insects (Oliver et al. 2010, Noda et al. 2001, Zchori-Fein and Brown 2002, Gomez-Valero et al. 2004, Tsuchida et al. 2002, Hail et al. 2011, Subandiyah et al. 2000), through cannibalism and blood contact between injured individuals in isopods (Le Clec'h et al. 2013, Rigaud and Juchault 1995), and presumably via predation, cannibalism, or paternal means in some North American funnel-web spiders (Baldo et al. 2008). In some hematophagous insects, namely certain species of mosquitoes and tsetse flies, HT of facultative *Wolbachia* occurs mainly via sexual transfer from an infected male to uninfected female. It is in these examples where *Wolbachia* is a secondary symbiont that often induces cytoplasmic incompatibility (CI) that results in the arrest of developing embryos, if the female does not possess molecular mechanisms for rescue (Brelsfoard et al. 2014, Werren et al. 2008).

To the best of our knowledge, HT of *w*Cle in *C. lectularius*, either through sexual or environmental means, has not been evaluated thus far. However, sexual transmission of surface (environmental) microbes does occur in bed bugs, perhaps not surprisingly, in association with their unusual copulation. During traumatic insemination the male paramere punctures the female cuticle and can transfer opportunistic pathogens from the paramere to the female's hemocoel (Reinhardt et al. 2005). Some of these sexually transmitted bacteria and fungi may cause disease and reduced survivorship in females (Reinhardt et al. 2005, Morrow and Arnqvist 2003).

In this study, we were interested in the possibility of HT of *w*Cle between mating adults (sexual) and to the progeny of *w*Cle-free females that mated with normal males. We used an established line of *C. lectularius* cleared of *Wolbachia*. We quantified *w*Cle16S rRNA copy number with droplet digital PCR. Our results indicate minimal HT of *w*Cle between adults, but suggest that *w*Cle-infected males can horizontally transmit *Wolbachia* to their progeny when mated to *w*Cle-free females.

Materials and Methods

Insects. The Winston-Salem (WS) strain was chosen for this experiment. Bed bugs were originally collected in 2008 from an infested apartment in Winston-Salem, NC, USA, and the colony has been maintained at North Carolina State University on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA). Bed bugs were maintained in small plastic containers on pleated card-stock shelters which contacted plankton netting on the top of the container through which bed bugs could feed. Blood was placed in a water-jacketed custom-made glass feeder and warmed to 38°C with a thermal circulator. A *Wolbachia*-free [Wb^{-}] colony of the WS strain was established using blood supplemented with antibiotics and B vitamins approximately 1 yr prior to this experiment. The Wb^{-} colony was fed weekly on defibrinated rabbit blood supplemented with rifampicin (10 µg/ml blood) and the Kao and Michayluk B Vitamin Solution (10 µl/ml blood) (Sigma Aldrich, St. Louis, MO) as adapted from Hosokawa et al. (2010). A colony of Wb^{-} individuals was later removed from antibiotics and maintained on B vitamins only. Both bed bug lines were reared at 27 ± 1°C (Thermo Scientific, Precision™ model#3727, Waltham, MA, USA) under a photocycle of

12/12 (light/dark); the Wb^- colony was maintained in a separate, dedicated incubator, with rearing containers inside secondary containment. The normal WS colony served as the Wb^+ group. Wb^- individuals for this experiment were used ~60 d after the colony ceased receiving antibiotics.

Experimental procedures. We investigated whether HT of *w*Cle can occur in *C. lectularius* using the field-collected WS strain. We examined sexual HT between cohabiting adults (female to male; male to female; and female to male to female), and social HT between cohabiting adults with eggs and hatching nymphs (female to eggs/nymphs; male to eggs/nymphs). Three treatment groups were set up with five replicates of each treatment. In each replicate, a single virgin individual of each sex was placed in a 7 ml glass vial with a screen-mesh cap and pleated card-stock. The treatments were: Wb^+Female→Wb^-Male; Wb^+Male→Wb^-Female; Wb^+Female→Wb^-Male→Wb^-Female. In the latter treatment a Wb^+ virgin female and a Wb^- virgin female were concurrently housed with a Wb^- virgin male, under the hypothesis that *Wolbachia* could be sexually transmitted to the male and then to the *Wolbachia*-free female. Importantly, the two females in each vial were not marked and could not be distinguished except post facto based on their respective *Wolbachia* titers. The number of eggs per female, and number of each neonate stage and mortality were recorded after each weekly feeding. Treatment vials were maintained for 30 d under similar conditions as colonies, with adults in contact with eggs and nymphs, and offered defibrinated rabbit blood supplemented with B vitamins once weekly.

DNA extraction. Total genomic DNA was extracted from all adults (n =45) and nymphs (n =41) using the DNeasy Blood and Tissue kit (QIAGEN, Germantown, MD, USA)

using a modified purification of total DNA from animal tissues (spin-column) protocol. Heads were removed from adults, and individual bed bugs were placed in 1.5 ml microcentrifuge tubes with 180 µl of ATL buffer solution and homogenized using a sterile plastic pestle. Proteinase K (20 µl) and 4 µl of RNase was immediately added after homogenization, and samples were then digested overnight (~24 h) in a 56°C water bath. Samples were then vortexed for 15 s, 200 µl of AL buffer was added, and incubated in a 70°C water bath for 10 min. Following incubation, samples were vortexed for 15 s, and 200 µl of 96% EtOH was added. The mixture was then pipetted onto the spin column, and the DNA was bound, washed, and eluted into 200 µl of AE buffer as outlined in the manufacturer's protocol with an additional rinse of AW2 buffer to further remove salts.

Verification of *Wolbachia*-free bed bugs. Conventional PCR was performed on individuals from the Wb^{-} colony, as well as from a colony removed from antibiotics after 30 and 60 d and maintained on B vitamins only. We measured presence or absence of *w*Cle using *Wolbachia*-specific primers (INTF2-FWD and INTR2-REV) adopted from Sakamoto and Rasgon (2006), which targeted a region of the *Wolbachia* 16S rRNA gene that produced a 136 bp amplicon. The GoTaq Green Master Mix (Promega, Madison, WI, P/N M7122) and nuclease-free water were used for all reactions at the following concentrations and volumes: 12.5 µl of 2x Master Mix, 2.5 µl of 10 µM INTF2-FWD, 2.5 µl of 10 µM INTR2-REV, 5 µl of template DNA, and PCR-grade, nuclease-free water was added to achieve a final reaction volume of 25 µl. Reactions were performed using an MJ Research thermocycler (model PTC 200, Bio-Rad Laboratories, Hercules, CA) with the following protocol: 95 °C for 2 min (95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min) x 36 cycles, and 72°C for 5 min. A no-template

control was used in the PCR reactions as well. A 2.0% agarose gel was used to confirm the 136 bp amplicon using a 100 bp DNA ladder and GelRed nucleic acid stain (Biotium, Hayward, CA), and visualized with a ChemiDoc-It TS2 imaging system (UVP, Upland, CA).

Quantification of *Wolbachia.* Absolute quantification of *Wolbachia* in each individual bed bug, was conducted using a ddPCR system (Model QX200, Bio-Rad Laboratories, Hercules, CA, USA). ddPCR was conducted on individuals from the Wb^{-} colony, as well as from a colony removed from antibiotics after 30, 60 and 90 d and maintained on B vitamins only. Bed bug DNA was combined with the *Wolbachia*-specific primers, TaqMan probes, and the ddPCR Supermix for Probes (Bio-Rad) into PCR-ready samples. Ribosomal protein RPL18 was used as the reference gene due to its stability (Mamidala et al. 2011), and RPL18 primers specific to *C. lectularius* produced a 137 bp amplicon. Double-quenched TaqMan probes with a 5' FAM fluorophore for *Wolbachia*, a 5' HEX fluorophore for *C. lectularius*, and 3' Iowa Black® FQ quenchers with internal ZEN quenchers (Integrated DNA Technologies, Inc., Coralville, IA, USA) specific to each target. Primer and probe sequences are listed in Table 2.1.

The ddPCR reaction was optimized using extracted bed bug DNA from Wb^{+} and Wb^{-} colonies. The bed bug/*Wolbachia* ddPCR assay comprised 22 µl of 1 x Droplet Supermix (Bio-Rad), 5 µl of genomic DNA isolated from a bed bug, 2 U of *Mse*I restriction enzyme (New England Biolabs, Ipswich, MA, USA), 500 nM each of forward and reverse primers and 250 nM each of FAM- or HEX-labeled TaqMan probes for bed bug and *Wolbachia* sequences, respectively. Then the 22 µl of PCR mixture was partitioned into an emulsion of ~20,000 droplets (each droplet = 1 nl) using a QX200™ AutoDG Droplet Digital PCR™

system (Bio-Rad). PCR was performed on a T100 Thermal Cycler using the following protocol: 95°C for 10 min and (94°C for 30s, 56°C for 2 min) x 40 cycles, and 98°C for 10 min. Post PCR, droplets were analyzed on the QX200 Droplet Reader. Absolute DNA copy numbers of bed bug and *Wolbachia* sequences in a sample were calculated on the Poisson distribution using the Quantasoft software version 1.7.4 (Bio-Rad). A bed bug DNA sample containing *w*Cle and a bed bug DNA sample Wb^{-} were included in each experiment to examine the variation in absolute DNA copy numbers of bed bug and *Wolbachia* between experiments. A no-template control was also included in each experiment to ensure no non-specific amplifications. To determine the quantification capacity of the ddPCR assay, serial dilutions of a DNA sample were prepared in water (5-, 25-, 125-, 625-, 3125-, and 15625-fold dilutions).

Quantitative analysis of differences in *Wolbachia* abundance. We generated descriptive statistics in SPSS Version 19 (IBM Corp., Armonk, NY, USA). We also used a General Linear Model (GLM) Univariate Analysis of Variance (ANOVA) and Tukey's HSD ($\alpha = 0.05$) in SPSS to determine significant differences in *Wolbachia* 16S copy number (mean ± SE) per bed bug. Samples with low DNA yield (<15 copies of the bed bug reference gene RPL18/µl) were not included in the analysis.

Results

The conventional PCR results confirmed that the bed bug colony treated with rifampicin and supplemented with B vitamins contained no *Wolbachia*. As well, the colony removed from antibiotics and maintained on vitamins only contained no *Wolbachia* (Fig. 3.1). The ddPCR

also confirmed the absence of *Wolbachia*. Absolute quantification detected 0 copies of the 16S *Wolbachia* target in both bed bug colonies treated with antibiotics and those later removed from antibiotics for 90 d (Fig. 2.1B). The dot spectrum (green) in quadrant IV indicates the RPL18 reference gene, the dot spectrum (gray) in quadrant III indicates neither *Wolbachia* or RPL18 target (Fig. 2.1A, B, C). In Fig. 2.1B, the absence of a dot spectrum (blue) in quadrant II indicates zero copies of *Wolbachia* 16S DNA detected in the sample. The ddPCR was highly sensitive for detection of *Wolbachia* and bed bug DNA. Theoretical values (1.76 copies of *Wolbachia* DNA/µl; 1.00 copies of RPL18 DNA/µl) and measured values (1.40 copies of *Wolbachia* DNA/µl; 1.00 copies of RPL18 DNA/µl) matched well with high reproducibility even at extremely low concentrations (15,625-fold dilution). No *Wolbachia* or bed bug DNA was detected in the no-template control.

In each group of Wb^{-} adults paired with Wb^{+} adults, mating and fecundity appeared normal, with the exception of one replicate in the $Wb^{+}M \rightarrow Wb^{-}F$ where adults did not ingest blood and no eggs were laid throughout the experiment. Table 3.1 shows the mean (± SE) number of eggs per female, per week from all five replicates, as well mean (± SE) number of each nymph stage at the end of the 30 d period. The $Wb^{+}F \rightarrow Wb^{-}M \rightarrow Wb^{-}F$ group produced the least number of eggs per female, and the $Wb^{+}F \rightarrow Wb^{-}M$ group produced the most, with ~30% more eggs per female than the other two groups, but the number of eggs produced per female was not significantly different among the three groups ($F_{2, 57} = 1.75$; $P = 0.182$). No adult mortality was observed, but one 2nd instar died in the $Wb^{+}F \rightarrow Wb^{-}M \rightarrow Wb^{-}F$ group at week 3. Overall, we saw no compelling evidence that the *Wolbachia*-free status of females or

males, or the direction of *w*Cle potential transmission affected adult fecundity or viability of the offspring.

Single adult female-male pairs were maintained for 30 d before they were extracted and *Wolbachia* quantified. Significant differences in the amount of *w*Cle per adult bed bug were observed between each group (Table 3.2). Overall, we saw minimal *Wolbachia* sexual transmission between adults. Wb^+ females and Wb^+ males (i.e., "donors") contained approximately equal amounts of *Wolbachia*, with the high mean (in Wb^+M) within 17% of the low mean (in Wb^+F). In the $Wb^+F \rightarrow Wb^-M$ group, *Wolbachia* was detected in only one of five Wb^- males, representing on average only 0.027% of the *Wolbachia* found in the Wb^+ females they mated with ($F_{1, 8}$= 64.183; $P < 0.001$). Likewise, only one of five Wb^-F female in the $Wb^+M \rightarrow Wb^-F$ group had detectable *Wolbachia*, which represented only 0.021% of the *Wolbachia* in the Wb^+ males they mated with ($F_{1, 7}$ = 38.186; $P = 0.001$). In the predicted sequential HT experiment with $Wb^+F \rightarrow Wb^-M \rightarrow Wb^-F$, no *Wolbachia* was detected in any of the males, and *Wolbachia* was detected from only five of the ten females ($F_{2, 11}$ = 132.25; $P < 0.001$). The amount of bed bug DNA varied much more than *Wolbachia* among individual male and female bed bugs, but was always at tens of thousands copies (Table 3.2).

Nymphs were present in all three groups, with the exception of one replicate in the $Wb^+M \rightarrow Wb^-F$ group where no eggs were produced. At week 4, no significant differences were observed in the number of 1st or 2nd instars among the three groups (Table 3.1), but the mean (± SE) number of 3rd instars in the $Wb^+M \rightarrow Wb^-F$ group was significantly lower than the $Wb^+F \rightarrow Wb^-M$ group ($F_{1, 8}$ = 20.59; $P = 0.0019$). The number of 3rd instars was also significantly different in the $Wb^+F \rightarrow Wb^-M \rightarrow Wb^-F$ group compared to the $Wb^+F \rightarrow Wb^-M$

($F_{1, 8}$ = 10.18; P = 0.0128). The number of 3rd instars in the $Wb^{+}M \rightarrow Wb^{-}F$ was not significantly different than those in the $Wb^{+}F \rightarrow Wb^{-}M \rightarrow Wb^{-}F$ group ($F_{1, 8}$ = 2.28; P = 0.1696). Overall however, as indicated above, all treatments produced viable offspring.

Because of poor DNA extractions, only progeny from the $Wb^{+}M \rightarrow Wb^{-}F$ group could be analyzed. We first modified the DNA elution step by eluting 2x with 50 µl each for a total volume of 100 µl of extracted DNA with fresh reagents, which resulted in more robust DNA extractions. *Wolbachia* was detected in 33 of 41 2nd and 3rd instars, and the mean (± SE) copy numbers of *Wolbachia* 16S rRNA and RPL18 detected are shown in Table 3.3. *Wolbachia* in nymphs represented 2.9% of the *Wolbachia* in their fathers and 135-fold the amount of *Wolbachia* in their mother (Tables 3.2, 3.3).

Discussion

The results presented here suggest that horizontal transmission (HT) of endosymbiotic *Wolbachia* in the common bed bug, *Cimex lectularius* is possible, but more research is needed to confirm and extend these findings. Our results suggest that *Wolbachia* could not re-establish in *Wolbachia*-free (Wb^{-}) adults after copulation with *Wolbachia*-infected (Wb^{+}) mates. At the same time however, Wb^{+} males appeared capable of transferring the endosymbiont to their progeny, either through the fertilized eggs of the Wb^{-} mothers or through subsequent contact of the nymphs with the male or his feces. Overall, we observed that (1) Wb^{+} and Wb^{-} adults mated normally; (2) Wb^{+} and Wb^{-} mating pairs produced similar numbers of viable offspring; (3) fecundity was reduced by 30% in Wb^{-} females in single female-male pairs; (4) *Wolbachia* did not reestablish in Wb^{-} adults through HT from

Wb^+ adults of the opposite sex during mating, even after 30 d of co-habitation, as indicated by significant differences in copy number of the 16S rRNA gene between the two; and (5) relatively low but significant amounts of *Wolbachia* were detected in 80% of the nymphs produced by Wb^- female that mated with Wb^+ males. These findings support the unusual conclusion that *Wolbachia* may be horizontally transmitted from male bed bugs to their offspring, but at the same time not infecting the adult female with *Wolbachia*. Nonetheless, the lack of sexual transmission to the female should be viewed with caution because *Wolbachia* may require one or more generations after the initial HT event to become established in the germline. *Wolbachia* may thus be considered a symbiont capable of mixed-mode transmission (MMT) in *C. lectularius*.

The lack of reestablishment of *w*Cle by way of sexual HT in adults is not surprising given the co-evolution and VT of *Wolbachia* in *C. lectularius* (Nikoh et al. 2014). The inability of *Wolbachia* to reestablish in *Wolbachia*-free males, even after multiple matings with Wb^+ females, is perhaps not unexpected due to the limited fluid from the female's paragenital tract that may enter the male's paramere and ultimately enter his hemocoel and the bacteriome. Likewise, paternal transmission of *Wolbachia* is rare in insects since it is largely excluded from mature sperm (Serbus et al. 2008). Transmission rates of *Wolbachia* through the male germline occur only 2% of the time in *Drosophila* (Serbus et al. 2008).

Two features of the bed bug however, might favor paternal transmission of *Wolbachia*. First, because traumatic insemination efficiently delivers sperm to the female's hemocoel, it is conceivable that a Wb^+ male might deliver *Wolbachia* to the hemocoel of a *Wolbachia*-free female. Second, recent evidence suggests substantial male mitochondrial

leakage to the fertilized egg, resulting in mitochondrial heteroplasmy (Robison et al. 2015). It is conceivable that if *Wolbachia* occurs in sperm cytoplasm and experiences similar dispersal as paternal mitochondria, it might escape ubiquitination and other processes that normally exclude sperm cytoplasmic components from entering the egg. Nevertheless, we detected only 35 ± 35 copies of the *Wolbachia* 16S rRNA gene in the Wb^- females that mated with Wb^+ males and co-habitated with them for 30 d, where *Wolbachia* DNA was detected in only 1 of 4 females (Table 3.2). It is possible that this low *Wolbachia* titer resulted from the mounting of an immune response in the female. Repeated matings through traumatic insemination significantly reduce the female's fitness due to injury, infection, and the mounting of an immune response (Stutt and Siva-Jothy 2001). The female has evolved adaptations of the spermalege organ to counter the fitness cost of mating (Morrow and Arnqvist 2003, Reinhardt et al. 2003; Benoit et al. 2016). The mesospermalege has an overabundance of hemocytes (Moriyama et al. 2012, Reinhardt and Siva-Jothy 2007), and the lipoprotein apolipophorin III which binds to the cell surface of microorganisms to stimulate the innate immune response, is found throughout the body of *C. lectularius* (Moriyama et al. 2012). Moreover, unlike in many other insects, where *Wolbachia* is widely and systemically distributed in various tissues such as fat body cells (Douglas 2015, Feldhaar 2011, Kikuchi 2009, Dale and Moran 2006), *w*Cle is highly localized in gonad-associated bacteriomes (Hosokawa et al. 2010). Although lysozyme gene expression is absent in both male and female bacteriomes of *C. lectularius* (Moriyama et al. 2012), it is possible that when *Wolbachia* is outside the bacteriome or gonads it is rapidly attacked by the immune system. The demanding transit through the hemocoel to the bacteriome may prevent paternal

Wolbachia from inoculating the female. Interestingly, several antimicrobial peptide genes are expressed in the bacteriomes, but it is unknown what specifically they recognize and whether mating induces expression of these genes (Moriyama et al. 2012).

Of particular interest is the detection of *Wolbachia* in the progeny of Wb^+ males and *Wolbachia*-free females. Paternal transfer of symbiotic bacteria has been demonstrated in mosquitoes and aphids (Damiani et al. 2008, Moran and Dunbar 2006). Male *Anopheles stephensi* have the bacteria *Asaia* in their reproductive organ, and can paternally transfer the bacteria to progeny. In the pea aphid *Acrythosiphon pisum*, the beneficial facultative symbionts *Regiella insecticola* and *Hamiltonella defensa* can be transferred sexually to females, not in sperm but in seminal fluids (Moran and Dunbar 2006). However, the efficiency of paternal transfer ranged from 0-100%, and both paternal *R. insecticola* and *H. defensa* infections were lost by generation 10 (Moran and Dunbar 2006). In the *w*Cle-*C. lectularius* association, how can *Wolbachia* be detected in larger amounts in 2^{nd} and 3^{rd} instar nymphs than in their mother? First, it is important to note that the amounts of *Wolbachia* DNA in these nymphs were much lower than amounts detected in Wb^+ 1^{st} instar *C. lectularius* (though of another strain) (Fisher et al. unpublished data), counter to expectations of an increasing amount of *Wolbachia* with development (Chapter 2, Table 2.2). Indeed, *Wolbachia* was not detected in 8 of the 41 nymphs we examined and the copy number of *Wolbachia* 16S rRNA was <100 per bed bug in 50% of the *Wolbachia*-positive nymphs.

The lack of sexual transmission of *Wolbachia* from males to females and its apparent transmission from males to offspring appears incongruous. It is possible however that while male sperm- or seminal fluid-associated *Wolbachia* are destroyed by the female's immune

system, some *Wolbachia* either bypass the female's hemocoel or survive the attack. These bacteria somehow infect the egg during fertilization, but fail to infect the female. Alternatively, it is plausible that *Wolbachia* was transmitted to nymphs not through the male germline, but through the male's feces. Nymphs of many insects, including hematophagous hemipterans, engage in coprophagy that facilitates their infection with a gut microbiome and possibly with *Wolbachia* as well. Triatomines do not harbor their endosymbionts in a specialized structure, and neonates acquire bacterial symbionts via coprophagy of adult feces (Schaub et al. 1989, Baines 1956). Facultative *Wolbachia* has been detected in the feces of the triatomine *Rhodnius pallescens*, as well as the salivary glands, gonads, and intestine (Espino et al. 2009). Likewise the endosymbiont *Blattabacterium* of cockroaches is found mainly in the fat body, but is also detected in feces (M.L. Kakumanu and C. Schal, unpublished data); although it is maternally transmitted vertically, neonates engage in coprophagy and could be inoculated with *Blattabacterium*. Early instar *C. lectularius* could potentially acquire *Wolbachia* post-oviposition through either coprophagy or other social transmission routes. It is interesting nonetheless that *Wolbachia*-free adult males and females were not inoculated with *Wolbachia* from feces, suggesting that if coprophagy is involved, it may be stage-specific. In other insects that acquire bacterial symbionts via HT, the likelihood of HT increases with host density, and VT increases with low host density where host fecundity is high (Ebert 2013). It is possible that we inadvertently created a situation that minimized HT by housing a single female and male in a vial. It would be interesting to know if HT is more prominent in crowded aggregations which better represent the natural habitat of bed bugs.

It is possible that *C. lectularius* has evolved efficient barriers to HT of *Wolbachia* to prevent cytoplasmic incompatibility (CI). While HT of certain strains of *Wolbachia* has long been known to cause CI in mosquitoes and *Drosophila* (Stouthamer et al. 1999, Serbus et al. 2008), CI has not been documented in bed bugs. In the genus *Glossina*, natural populations are regularly co-infected with 3 symbionts; the obligate mutualist *Wigglesworthia*, commensal *Sodalis*, and parasitic *Wolbachia* (Weiss et al. 2011). *Wigglesworthia* and *Sodalis* are vertically transmitted via maternal milk gland secretions to intrauterine larvae, but *Wolbachia* resides in the gonads and transmitted to offspring during oogenesis (Aksoy et al. 2013). Several haplotypes of *Wolbachia* infect *Glossina* (Symula et al. 2013), where some cause CI, while other strains do not, and spread through natural populations of tsetse flies without detrimental effects on sex ratios (Aksoy et al. 2013). It is presumed that only one strain of *Wolbachia* occurs in *C. lectularius* (Rasgon and Scott 2004), and VT through a maternal lineage might ensure that other haplotypes are excluded and potential CI circumvented.

Obligate symbiotic microorganisms of insects positively influence the fitness of their host, in most cases contributing essential micronutrients otherwise lacking in the host's restricted diet (Douglas 2015, Douglas 2009). Evolutionary concepts and relationships have been developed for symbionts with exclusive VT or HT (Ebert 2013). In certain symbiotic associations, the propensity of a symbiont for VT or HT is plastic, where the pathway is dependent on abiotic factors, and the expected reproductive and survival rates of the host (Ebert 2013). It has been accepted that *w*Cle, as an obligate nutritional mutualist with *C. lectularius*, has exclusive VT from mother to oocytes (Nikoh et al. 2014). This is further

supported by the primary concentration of *Wolbachia* in the gonad-associated bacteriome of the female. But if the male host is an "evolutionary dead end" for *Wolbachia*, why would *w*Cle also concentrate exclusively in the bacteriome of the male? Does *Wolbachia* enter spermatocytes during spermatogenesis? Is it then excluded from mature sperm, as in other insects? If *Wolbachia* is in bed bug sperm, are there opportunities for multiple haplotypes and potential CI? The main metabolic tissue in insects is the fat body, so why would *w*Cle, a nutritional mutualist, not associate with the fat body? Our results invite new hypotheses to further explore the bed bug-*w*Cle evolutionary relationship, and evaluate the potential and extent of HT in other species in Cimicidae. Furthermore, the results presented here underscore the need for experimental research into the mechanisms that prevent sexual transmission in bed bugs and potentially favor the acquisition of *Wolbachia* by coprophagy. Finally, bed bugs offer a rich system in which to investigate the dynamic balance between the evolution of host manipulation by *Wolbachia* (male killing or feminization, CI, parthenogenesis), which would favor sexual, social and environmental HT, and mutualism, which would favor strict vertical transmission of *Wolbachia*. The coupling of obligate mutualism with extensive inbreeding in bed bugs is expected to select against sexual transmission of *Wolbachia*. Yet recent findings of obligate mutualism and reproductive manipulation within the same host-symbiont association call for caution in predicting evolutionary trajectories of *Wolbachia*-host associations.

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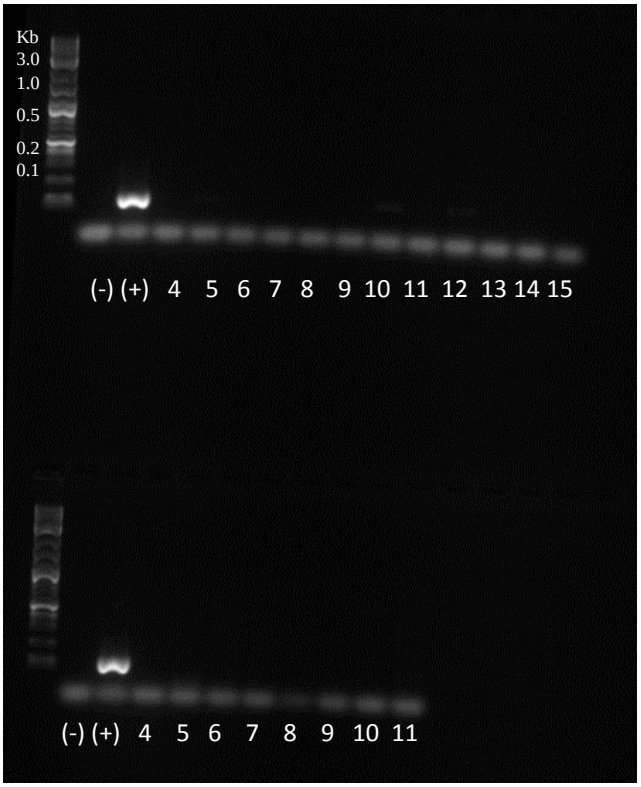

Figure 3.1. PCR results illustrating absence of *Wolbachia* in bed bugs. Top row: lanes 4-9: bed bugs treated with the antibiotic + B vitamins, lanes 10-15: bed bugs removed from antibiotics and maintained on B vitamins only for 30 d; Bottom row: lanes 4-11: bed bugs removed from antibiotics and maintained on B vitamins only for 60 d.

Table 3.1. Offspring produced by three HT treatments. Mean (± SE) number of eggs per female per week, mean (± SE) total eggs per female produced, and mean (± SE) number of nymphs for each treatment group after 30 d. $N = 5$ for all groups. Different capital letters in parentheses indicate significant differences, respect to life stage (Tukey's HSD, $P < 0.05$).

	$Wb^+F \rightarrow Wb^-M$	$Wb^+M \rightarrow Wb^-F$ [a]	$Wb^+F \rightarrow Wb^-M \rightarrow Wb^-F$	
Eggs				Week
	9.8 ± 2.65	6.0 ± 2.21	6.9 ± 0.70	1
	4.2 ± 2.40	4.4 ± 1.96	3.6 ± 0.86	2
	7.8 ± 1.16	4.2 ± 1.93	3.4 ± 0.68	3
	4.2 ± 0.58	4.0 ± 1.45	4.2 ± 0.77	4
Total	**26.0 ± 6.79** (A)	**18.6 ± 7.55** (A)	**18.1 ± 3.00** (A)	
Nymphs				
1sts	6.4 ± 0.93 (A)	5.0 ± 1.76 (A)	3.9 ± 0.48 (A)	4
2nds	5.0 ± 1.00 (A)	6.0 ± 2.10 (A)	4.5 ± 1.14 (A)	4
3rds	9.2 ± 1.24 (A)	2.2 ± 0.92 (B)	4.2 ± 0.96 (B)	4

[a] one replicate had no blood-feeding, no apparent mating, and no egg production

Table 3.2. Horizontal transmission of *Wolbachia* between adult males and females mated and co-housed for 30 d. Mean copy number (± SE) of *Wolbachia* 16S rRNA gene per adult bed bug, mean copy number (± SE) of bed bug RPL18 gene, and ratio (± SE) of *Wolbachia* 16S rRNA gene to bed bug RPL18 gene. $N = 5$, except where indicated, for each treatment group. Samples represent adult bed bugs extracted 30 d after the respective treatments were paired.

	$Wb^+F \rightarrow Wb^-M$		$Wb^+M \rightarrow Wb^-F$		$Wb^+F \rightarrow Wb^-M \rightarrow Wb^-F$		
	Female	Male	Male	Female	Female 1	Male	Female 2
Wb positive	5 of 5	1 of 5	5 of 5	1 of 4	5 of 5	0 of 5	0 of 5
Wolbachia 16S rRNA	136,664 ± 90,546	37 ± 37	164,278 ± 72,332	35 ± 35	151,387 ± 28,667	0 ± 0[a]	0 ± 0
Bed bug RPL18	105,829 ± 48,222	66,370 ± 17,875	375,056 ± 27,439	100,474 ± 39,858[b]	33,475 ± 12,305	38,931 ± 9808	46,253 ± 12,138
Ratio	2.06 ± 1.08	0.0003 ± 0.0003	0.4575 ± 0.1994	0.0016 ± 0.0016	9.76 ± 4.45	0 ± 0	0 ± 0

[a] one replicate excluded due to ddPCR error

[b] one replicate excluded; < 15 copies bed bug DNA/µl

Table 3.3. Horizontal transmission of *Wolbachia* from Wb^+ males to their progeny. Mean copy number (± SE) of *Wolbachia* 16S rRNA gene per bed bug, mean copy number (± SE) of bed bug RPL18 gene, and ratio (± SE) of *Wolbachia* 16S rRNA gene to bed bug RPL18 gene in 41 nymphs from the $Wb^+M \rightarrow Wb^-F$ group.

	Nymphs
Wb positive	33 of 41
Wolbachia 16S rRNA	4745 ± 3572
Bed bug RPL18	41,465 ± 2158
Ratio	0.0953 ± 0.0639

CHAPTER 4.

Lack of Influence of *Wolbachia* Endosymbiont on Virus Titer in *Cimex lectularius*

Abstract

The world has seen a significant increase in bed bug infestations over the past 20 years, and resistance to insecticides, reintroductions in previously-treated areas, and increased global travel have all contributed to the increasing difficulty of controlling bed bug populations. Bed bugs, *Cimex lectularius*, are obligatory blood-feeding ectoparasites that require a blood meal to molt and produce eggs. Frequent blood meals and their intimate association with humans increase the potential for disease transmission. However, despite more than 100 years of inquiry into bed bugs as disease vectors, they still have not been conclusively linked to any disease or pathogen. This ecological niche is extraordinarily rare, given that nearly every other blood-feeding arthropod is associated with some type of human or zoonotic disease. The bacterial symbiont *Wolbachia* is found within an estimated 66% of arthropod species. It is the primary, obligate endosymbiont of the common bed bug, that biosynthesizes B vitamins for the nutritionally-deficient host, but it is unknown if it confers additional benefits to its bed bug host. In some insects, *Wolbachia* induces resistance to viruses such as Dengue, Chikungunya, West Nile, Drosophila C, and Zika, and primes the insect immune system in other blood-feeding insects. *Wolbachia* may adopt a similar role in its mutualistic association with the bed bug. The objective of this project was to evaluate the influence of *Wolbachia* on virus replication within *C. lectularius*. We fed an established line of *Wolbachia*-cured bed bugs and a *Wolbachia*-positive line a virus-laden blood meal that used feline calicivirus as the model, and quantified the amount of virus over five time intervals post-feeding. There was a significant effect of time as the amount of virus declined by ~90% over 10 days in both groups, but no significant difference in virus titer was observed between the *Wolbachia*-

positive and *Wolbachia*-free groups. Results suggest that other mechanisms are involved in virus suppression within bed bugs, independent of any influence of *Wolbachia*, and our conclusions underscore the need for future research.

Background

The common bed bug, *Cimex lectularius*, is an obligate blood-feeding ectoparasite that has undergone a global resurgence in the last two decades (Benoit et al. 2016, Wang et al. 2013, Romero et al. 2007, Potter 2006, Doggett et al. 2004). The recent discovery of new mechanisms of resistance (Romero et al. 2016, Lilly et al. 2016a, b, Koganemaru et al. 2013) makes bed bug infestations increasingly difficult to control, but the significant fitness costs associated with resistance could be exploited in integrated pest management plans (Gordon et al. 2015). Highly specialized treatments which are cost-prohibitive to most low-income residents, coupled with insecticide resistance exacerbate the resurgence by often failing to prevent reintroductions (Raab et al. 2016). This profound resurgence of bed bugs in such frequent associations with humans and our domiciles could increase the threat of disease transmission.

Bed bugs, like the related triatomine bugs that transmit Chagas disease, are hemimetabolous, so each instar and all adults require at least one blood-meal to develop and reproduce. Such frequent re-feeding could contribute substantially to their importance as disease vectors. Yet, despite being exclusively hematophagous, to date bed bugs have not been conclusively implicated in vector-borne disease transmission. Bed bugs can acquire a myriad of blood-borne pathogens from their hosts, but in the case of ingested viral particles, most viruses do not or are not capable of replicating once inside the bed bug (Delaunay et al. 2011, Goddard and deShazo 2009, Lai et al. 2016). Hepatitis B virus (HBV) is a notable exception, however. It has been detected >45 d post ingestion, after direct injection into the hemocoel, and HBV is stercorarially shed in bed bug feces, suggesting the possibility of

mechanical transmission (Blow et al. 2001, Delaunay et al. 2011, Silverman et al. 2001), if HBV can enter and replicate in the hemocoel. Recently, bed bugs have been shown to experimentally acquire, maintain, and effectively transmit *Trypanosoma cruzi*, the etiologic agent of Chagas disease (Salazar et al. 2015), and *Bartonella quintana*, the etiologic agent of Trench fever (Leulmi et al. 2015), but a survey of field-collected bed bugs failed to detect *Bartonella* (Saenz et al. 2013). Nevertheless, the latter survey detected *Burkholderia multivorans* in bed bugs.

Microbe-microbe interactions with respect to pathogen suppression have been studied in various blood-feeding insects such as the kissing bug *Rhodnius* (Azambuja et al. 2005, Beard et al. 2001), tsetse fly *Glossina* (Weiss et al. 2013, Weiss and Aksoy 2011), and mosquitoes, and also in plant-feeding fruit flies and aphids. The insect microbiome can modulate vector competence of the host for arboviruses (Johnson 2015a, Jupatanakul et al. 2014, Weiss and Aksoy 2011), and these influences have been most often evaluated in associations of the endosymbiont *Wolbachia* with fruit flies and mosquitoes. *Wolbachia* protects *Drosophila* against virus-induced mortality for Drosophila C virus (DCV) and Flock house virus (FHV) (Teixeira et al. 2008, Wong et al. 2011). *Wolbachia* also stimulates immune gene expression in several mosquito species (Hughes et al. 2011, Kambris et al. 2010, 2009, McFarlane et al. 2014, Pan et al. 2012), and thus increases resistance to, and reduces the vector competence of *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* mosquitoes for viruses such as Dengue, Chikungunya, West Nile, and Zika (Aliota et al. 2016, Bian et al. 2010, Glaser and Meola 2011, Moreira et al. 2009, Mousson et al. 2012, Zhang et al. 2013). Dengue, Chikungunya, Zika, West Nile, DCV, and FHV are all

positive-sense single-stranded RNA viruses (+ssRNA), suggesting that the anti-viral effects induced by *Wolbachia* in *Drosophila* and mosquitoes might be limited to RNA viruses (Brownlie and Johnson 2009, Sicard et al. 2014).

Cimex lectularius harbors *Wolbachia* as its primary endosymbiotic nutritional mutualist that biosynthesizes B vitamins for its nutritionally deficient host (Hosokawa et al. 2010, Moriyama et al. 2015) in a co-dependent relationship that has presumably evolved over several million years (Nikoh et al. 2014). Additional fitness benefits that the endosymbiont might confer upon bed bugs have not been investigated. Similar to the effects reported in other arthropods, *Wolbachia* could influence the vector competence of *C. lectularius* through mechanisms involving interactions with the host or with ingested pathogens, thus preventing certain viruses from replicating within the host. This may explain in part why bed bugs are not a major disease vector for arboviruses. The objective of this study was to compare virus titer in *Wolbachia*-positive vs. *Wolbachia*-free *C. lectularius* at several time intervals after ingesting a virus-laden blood meal.

Methods

Establishment of *Wolbachia*-free *C. lectularius* colonies

The Winston-Salem (WS) strain of *C. lectularius* was collected in Winston Salem, NC in 2008 and fed defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) in an artificial feeding system, as described by Sierras and Schal (2017). Ten adult males and 20 adult females of the WS strain were divided equally and placed into two separate 20 ml glass vials with screened-caps and a creased length of card stock for harborage. These vials were then

placed into a plastic container (17.8 x 12.7 x 10.2 cm) as additional protection against environmental bacteria. A semi-sterile incubator (Thermo Scientific, Precision Model #3727, Waltham MA) was dedicated to rearing the *Wolbachia*-free (Wb^{-}) colonies and it was maintained at 27 °C and a photoperiod of 12:12 (light:dark [L:D]). These two colonies were fed weekly on defibrinated rabbit blood supplemented with the antibiotic rifampicin (10 µg/ml blood) and the Kao and Michayluk B Vitamin Solution (10 µl/ml blood) (Sigma Aldrich, St. Louis, MO) as adapted from Hosokawa et al. (2010). Rearing vial (jar) size was increased periodically as colony numbers increased. To further mitigate external environmental contaminants, vials of blood were put under a portable UV light for 5 min immediately prior to feeding, and glass water jacket feeders were washed with detergent and boiled for 5 min after each weekly feeding.

Extraction of genomic DNA from *C. lectularius*

To verify antibiotic-vitamin treated colonies of *C. lectularius* were free of *Wolbachia*, a comparison to the WS-Wb^{+} normal strain was conducted. After several filial generations, six adults were randomly selected from each of the two antibiotic-vitamin supplemented colony jars (n=12) and six from the untreated WS strain. Total genomic DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN, Germantown, MD) with a modified purification of total DNA from animal tissues (spin-column) manufacturer's protocol. Individual bed bugs with heads removed were homogenized in the 1.5 ml microcentrifuge tube using a sterile plastic pestle and then digested overnight (~24 h) in 180 µl of ATL buffer solution, 20 µl of proteinase K, and 4 µl of RNase in a 56 °C water bath. Following initial digestion,

samples were vortexed for 15 s, 200 µl of AL buffer was added, and then incubated in a 70 °C water bath for 10 min. After incubation, 200 µl of 96% ethanol was added, the mixture was then pipetted onto the DNeasy Mini spin column, and the DNA was bound, washed, and eluted into 200 µl of AE buffer as outlined in the protocol. An additional wash with AW2 buffer was included to further remove salts. Samples were stored at -20 °C until PCR was conducted.

Verification of *Wolbachia*-free *C. lectularius*

Conventional PCR was conducted to amplify a specific gene target within *Wolbachia* and measure presence or absence of *Wolbachia.* The *Wolbachia*-specific primers adopted from Sakamoto and Rasgon (2006) (INTF2-FWD and INTR2-REV) targeted a region of the *Wolbachia* 16S rRNA gene that produced a 136 bp amplicon. The GoTaq Green Master Mix (Promega, Madison, WI, P/N M7122) and nuclease-free water were used for all reactions at the following concentrations and volumes: 12.5 µl of 2x Master Mix, 2.5 µl of 10 µM INTF2-FWD, 2.5 µl of 10 µM INTR2-REV, 5 µl of template DNA, and PCR-grade nuclease-free water was added to achieve a final reaction volume of 25 µl. Reactions were performed using an MJ Research thermocycler (model PTC 200, Bio-Rad Laboratories, Hercules, CA) with the following protocol: 95 °C for 2 min (95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min) x 36 cycles, and 72 °C for 5 min. A no-template control was used in the PCR reactions as well. A 2.0% agarose gel was used to separate the 136 bp amplicon using a 100 bp DNA ladder and GelRed nucleic acid stain (Biotium, Hayward, CA), and visualized with a ChemiDoc-It TS2 imaging system (UVP, Upland, CA).

To obtain absolute quantification of *Wolbachia* in each individual bed bug, a droplet digital PCR (ddPCR) system (Model QX200, Bio-Rad Laboratories, Hercules, CA, USA) was utilized. Bed bug DNA was combined with the *Wolbachia*-specific primers, TaqMan probes, and the ddPCR Supermix for Probes (Bio-Rad) into PCR-ready samples. Primers for a ribosomal protein (RPL18) specific to *C. lectularius* were used as the reference gene due to its stability (Mamidala et al. 2011); they produced a 137 bp amplicon. We used double-quenched TaqMan probes with a 5' FAM fluorophore for *Wolbachia*, a 5' HEX fluorophore for *C. lectularius*, and 3' Iowa Black® FQ quenchers with internal ZEN quenchers (Integrated DNA Technologies, Inc., Coralville, IA, USA) specific to each target. Primer and probe sequences are listed in Table 2.1.

The ddPCR reaction was optimized using extracted bed bug DNA from Wb^+ and Wb^- lines. The bed bug/*Wolbachia* ddPCR assay comprised 22 µl of 1 x Droplet Supermix (Bio-Rad), 5 µl of genomic DNA isolated from a bed bug, 2 U of *Mse*I restriction enzyme (New England Biolabs, Ipswich, MA), 500 nM each of forward and reverse primers and 250 nM each of FAM- or HEX-labeled TaqMan probes for bed bug and *Wolbachia* sequences, respectively. Then the 22 µl of PCR mixtures were partitioned into an emulsion of ~20,000 droplets using a QX200™ AutoDG Droplet Digital PCR™ system (Bio-Rad). PCR was performed on a T100 Thermal Cycler using the following protocol: 95°C for 10 min and (94°C for 30s, 56°C for 2 min) x 40 cycles, and 98°C for 10 min. Post PCR, droplets were analyzed on the QX200 Droplet Reader. Absolute DNA copy numbers of bed bug and *Wolbachia* sequences in a sample were calculated on the Poisson distribution using the Quantasoft software version 1.7.4 (Bio-Rad). Previously confirmed Wb^+ bed bug DNA

sample and Wb^- bed bug DNA sample were included in each experiment as positive and negative controls. No-template control was also included in each experiment to ensure no non-specific amplifications. To determine the quantification capacity of the ddPCR assay, serial dilutions of a DNA sample (x5, x25, x125, x625, x3125, x15625) in water were prepared.

Virus inoculations and treatments

The experiment evaluated virus titers over time in three cohorts of bed bugs: control (Wb^+), antibiotic-vitamins (Wb^-), and Wb^- maintained for 90 d on vitamin-supplemented blood without antibiotic. The latter (vitamin-only) group was removed from antibiotic 90 d prior to inoculation with virus.

Feline calicivirus (FCV) was chosen as the inoculum due to its environmental stability and feasibility as a viral pathogen. Feline calicivirus is a (+)ssRNA virus that is one of the primary causes of respiratory infections in felines. Virus was grown in existing Crandell Reese Feline Kidney (CrFK) cell line at the North Carolina State University College of Veterinary Medicine Clinical Virology Laboratory and stored at -80 °C in 2 ml aliquots per established protocols. The FCV stock was produced by first removing the growth medium from confluent CrFK monolayers in 75 cm^2 cell culture flasks by aspiration and then 1 ml of the virus inoculum was added to each flask. Flasks were incubated at 37 °C in 5% CO^2 atmosphere for 90 min to allow for virus adsorption. Each flask then received 10 ml of maintenance medium (MEM-2% fetal bovine serum), and incubated 16 h at 37 °C in a 5% CO^2 which resulted in virus-induced destruction of ~90% of the monolayer.

In each feeding, 40 individual bed bugs were chosen randomly, placed in 7 ml glass vials with screened-caps, and fed as previously described for 15 min on fresh defibrinated rabbit blood supplemented with 1 ml FCV (10^7 $CCID_{50}$/ml) per 1 ml of blood. $CCID_{50}$ is the 50% cell culture infective dose, as defined below. Bed bugs were starved 7 days prior to feeding FCV-laden blood. Individuals that did not feed or only partially fed were removed and discarded. Vials were kept thereafter at room temperature under a sterile laminar flow hood and 12:12 (L:D) photoperiod.

Quantification of FCV in *C. lectularius*

Bed bugs were killed at the following time intervals: 5 hrs, 24 hrs, 4 d, 7 d, and 10 d post feeding. Three randomly chosen bed bugs were removed from each cohort, sexed, and surface sterilized with 0.05% NaClO and 70% EtOH. Surface-sterilized bugs were homogenized in 3.5 ml round-bottom polystyrene tubes (Sarstedt, Nümbrecht, Germany) in 0.5 ml of Dulbecco's Modified Eagle's Medium (DMEM) (Caisson Laboratories, Smithfield, UT) with sterile plastic pestles and the tubes were centrifuged (8000 rpm for 1 min). A volume of 220 µl of the supernatant was pipetted into a new 3.5 ml tube with 2.2 ml of DMEM, and serial 10-fold dilutions (10^{-1} to 10^{-6}) were performed to obtain virus titration. A total of 100 µl of each dilution was placed in 4 wells (technical replicates; portrait orientation) of a flat-bottomed, 96-well plate, and 100 µl of CrFK cells was added to wells. Each plate contained a row of wells as a cell control with no virus. Plates were incubated at 37 °C for 5 d (Henzel et al. 2012, Bae and Schwab 2007, Bidawid et al. 2003) and stained with crystal violet (50 µl/well) where only live cells absorbed the stain.

In each virus dilution, the percentage of dead (infected) cells was visually determined for each well. To measure the infectious virus titer, the 50% cell culture infective dose ($CCID_{50}$) endpoint dilution assay was used to quantify the amount of virus required to kill 50% of infected CrFK cells as described by Reed and Muench (1938), if the 50% dose fell between two dilutions. The Reed-Muench index formula reads as follows:

$$\frac{\text{(\% infected at dilution immediately above 50\%) - 50\%}}{\text{(\% infected at dilution immediately above 50\%) - (\% infected at dilution immediately below 50\%)}}$$

Sex ratio of male and female *Cimex lectularius* chosen

The sex ratio of the three bed bugs randomly chosen per time interval post-feeding of FCV is shown in Table 4.1. Both Wb^- and Wb^- 90 d groups had close to 50% male:female, but slightly more males were chosen in the Wb^+ group at 60:40 male:female ratio.

Statistical analysis

Differences in the mean virus titer in $CCID_{50}$/ml were analyzed with a two-sample t-test that assumed unequal variances using a 95% confidence interval with SPSS Version 19 (IBM Corp., Armonk, NY). *P*-values < 0.05 were considered significantly different. A General Linear Model Repeated Measures analysis (Wilks' Lambda) was also conducted in SPSS to identify any effect of time, treatment group, replicate, and interactions between these variables on virus titer.

Results

Confirmation of *Wolbachia*-free *Cimex lectularius* colonies

The conventional PCR results confirmed that the bed bug colony treated with rifampicin and supplemented with B vitamins contained no *Wolbachia*. As well, the colony removed from antibiotics and maintained on vitamins only contained no *Wolbachia* (Fig. 3.1). The ddPCR also confirmed the absence of *Wolbachia*. Absolute quantification detected 0 copy numbers of the 16S *Wolbachia* target in both bed bug colonies treated with antibiotics and those later removed from antibiotics for 90 d (Fig. 2.1B). The dot spectrum (green) in quadrant IV indicates the RPL18 reference gene, the dot spectrum (gray) in quadrant III indicates neither *Wolbachia* or RPL18 target (Fig. 2.1A, B, C). In Fig. 2.1B, the absence of a dot spectrum (blue) in quadrant II indicates zero copy number of *Wolbachia* 16S detected in the sample. The ddPCR was highly sensitive for detection of *Wolbachia* and bed bug DNA. Theoretical values (1.76 copies of *Wolbachia* DNA/µl; 1.00 copies of RPL18 DNA/µl) and measured values (1.40 copies of *Wolbachia* DNA/µl; 1.00 copies of RPL18 DNA/µl) matched well with high reproducibility even at extremely low concentrations (15,625-fold dilution). No *Wolbachia* or bed bug DNA was detected in the no-template control.

FCV acquisition and titer comparison in bed bug groups

We estimated adult bed bugs ingested between 4-6 µl of blood meal, which correlates to 10^4 virus in a single feeding. Live virus was detected in relatively large amounts in all treatment groups at all time intervals up to 10 d (Table 4.2, Fig. 4.1). No significant differences were observed in FCV titer among the three treatment groups at any of the five sampling intervals,

with the exception of in the Wb^- group compared to the Wb^- 90 d at the 4 d interval ($t(2) = -4.724$, $P = 0.0179$)

There was a significant decline in FCV titer over time after the blood meal [Wilks' $\lambda = 0.014$, $F = 36.48$, $df = 4, 2$; $P = 0.0271$; $\eta^2 = 0.986$], with an average of 90.7% across all three treatments after 10 d, ranging from 96.5% decline in the Wb^+ group, 92.2% in the Wb^- group, and 83.4% decline in the Wb^- 90 d group (Table 4.2). However, there was no effect of treatment ($F = 0.359$, $df = 2$, $P = 0.575$) or replicate on FCV titer ($F = 0.127$, $df = 2$, $P = 0.884$), and there was no effect of time*treatment [Wilks' $\lambda = 0.072$, $F = 6.48$, $df = 4, 2$; $P = 0.1381$; $\eta^2 = 0.928$], or time*replicate [Wilks' $\lambda = 0.05$, $F = 1.73$; $df = 8, 4$; $P = 0.3143$; $\eta^2 = 0.775$]. No infection was observed in any of the cell line controls in any of the three bed bug groups at any sampling time interval. We observed no mortality in any of the FCV-infected bed bugs that were left in the vials after feeding on FCV-supplemented blood.

Discussion

In 1887 Elias Metschnikoff was the first to suspect that bed bugs could vector human pathogens, but definitive proof having been elusive, several generations of researchers remained unconvinced (Usinger 1966). Even today, bed bugs are not considered important vectors of any specific pathogen, although they are broadly considered medically-important due to the clinical manifestations of bite site reactions and the psychological effects that infestations can elicit (Goddard and deShazo 2012, 2009, Reinhardt and Siva-Jothy 2007). Although more than 45 pathogens (bacteria, viruses, fungi, protozoans) have been detected in bed bugs (Delaunay et al. 2011), <10% are known to replicate within the bed bug. Their

apparent refractory state to human pathogens is remarkable, as nearly every other blood-feeding arthropod (mosquitoes, biting flies, lice, fleas, ticks) is associated with some type of disease or pathogen, and it may reflect the intimate and long evolutionary association of bed bugs with humans.

We used feline calicivirus (FCV) as a model pathogen in our investigation because it is environmentally stable, it represents RNA viruses, and it could be transmitted by bed bugs in residential settings. Our results showed that FCV did not replicate within *Cimex lectularius*, but relatively high FCV titers were maintained in bed bugs 10 d after they were inoculated through a blood meal. The amount of FCV decreased dramatically over time in all of our treatment groups, from a 29% decline from 5 to 24 hrs after the blood meal, to a 91% decline after 10 days. We found no evidence from these patterns that FCV could replicate within the bed bug. Although we observed a large decline in FCV, the overall decline was much smaller than other viruses evaluated in bed bugs, such as HIV and Yellow Fever where little to no replication was reported to occur (Jupp and Lyons 1987, Burton 1963) Importantly however, the decline in FCV titer was independent of the presence or absence of endosymbiotic *Wolbachia* (Table 4.2, Fig. 4.1).

It is important to note that secondary effects from the rifampicin treatment could have affected the interaction of the bed bug with FCV. The *Wolbachia*-cured (Wb^{-}) group was treated with antibiotic during weekly blood meals before the experiment started. Therefore, this group was also expected to suffer from an altered gut microbiome, whose possible interactions with and effects on FCV are not known. Another *Wolbachia*-cured group (Wb^{-} 90 d) was weaned off rifampicin 3 months before the experiment started. We expected this

group to be less affected by the antibiotic, and its gut microbial community might have recovered during the 3 months, which represented >2 generations. This group, however, was not different from the other two groups, including the *Wolbachia*-containing group (Wb^+) never exposed to rifampicin, suggesting that neither *Wolbachia* nor the gut microbiome influenced the FCV titers.

The absence of a *Wolbachia* influence on FCV titers might be related to minimal interactions between the symbiont and the virus. Factors related to the physiological conditions of the midgut, inability of FCV to permeate midgut barriers, and host immune responses may minimize these interactions. *Wolbachia*'s intracellular sequestration within the bed bug bacteriome might further diminish contact between these two microbes.

As blood is ingested, FCV would interact with a wide range of bed bug salivary proteins that are secreted to counteract the vertebrate host's hemostasis (platelet aggregation, fibrin crosslinking, vasoconstriction, local immune responses). The bed bug genome revealed expanded families of salivary apyrases, nitric oxide carriers, and members of the Ap4a_hydrolase family (Benoit et al. 2016). Bed bug saliva contains substances that decrease ingested pathogen virulence and titers (Lai et al. 2016), but it is not known if viruses might be affected by these salivary components.

After the bed bug ingests a blood meal containing FCV, the virus must interact with the insect alimentary canal, penetrate the hemocoel, and for effective transmission with subsequent blood meals the virus needs to replicate in the salivary glands or other tissues associated with the mouthparts. Alternatively, if the virus survives passage through the alimentary canal, it can be transmitted in feces, though this pathway is considered less

efficient. Mildly acidic to neutral midgut pH (5-7) is ideal for a wide range of microorganisms, but in most insects the midgut is alkaline (pH ≥8) and typically unfavorable for most microorganisms (Douglas 2015, Harrison 2001). Adult mosquito midgut pH is between 7.2-7.9 immediately prior to bloodmeal ingestion, and returns to pH 7.3 after digestion (Chapman 2013). The gut pH of the bed bug is not known, but if it is similar to the mosquito, it likely is reasonably favorable to FCV. FCV is a non-enveloping, environmentally stable virus, able to survive acidic to neutral pH (Lee and Gillespie 1973). Therefore, FCV likely survives the midgut, although the activity of digestive enzymes may hinder FCV. The bed bug genome revealed 187 potential digestive enzymes, including serine proteases, a large expansion of cathepsin D genes, and aspartic proteases that are specifically adapted for acidic pH (Benoit et al. 2016).

We did not determine whether FCV was able to cross the midgut barrier and enter the bed bug hemocoel. Many insects, including blood feeders, form a peritrophic membrane (PM) around the food bolus during or shortly ingestion. The PM is a physical barrier that protects the midgut lumen. A pathogen or parasite must penetrate the PM and invade the midgut tissues before it can cross into the hemolymph. Although the PM is absent in majority of Hemiptera (Chapman 2013), *C. lectularius*, *R. prolixus*, and *Triatoma infestans* all have a modified PM effectively known as a 'plexiform surface coat' type PM (Peters 2012). The PM is permeable to digestive enzymes, and combined with other midgut effector mechanisms such as lectins, reactive oxygen species, nitric oxide, melanization through the prophenoloxidase cascade, and pattern recognition receptors, are all humoral immunity factors that benefit the host against infections (Ratcliffe and Whitten 2004). Several

pathogens of insects escape humoral response and evade the impermeability of the PM by invading the tissues before the PM is fully developed (Chapman 2013). The mosquito PM is impermeable to particles >148 kDa (Edwards and Jacobs-Lorena 2000), and most viruses are ~2000 kDa (Shao et al. 2001). To permeate through the PM, viruses and other enteric pathogens must secrete proteolytic enzymes that degrade membrane proteins (Shao et al. 2001). Once crossed into the hemolymph, most arboviruses infect all compartments of an arthropod vector (Ratcliffe and Whitten 2004).

If FCV crossed the PM into the hemolymph, the next host defense would be a systemic immune response by *C. lectularius* by way of hemocytes, activation of proteases, production of antimicrobial peptides, or immune signal transduction pathway activation (Toll, imd, JAK-STAT) by the fat body that could lower FCV titer. The genome of *C. lectularius* revealed members of all these pathways, as well as the RNA interference pathway (Benoit et al. 2016) and transcriptomic analysis supports the expression of the entire suite of putative immune defense pathways (Bai et al. 2011). It is generally thought that the combination of blood-feeding and traumatic insemination have selected for a highly adapted immune response in the bed bug. For example, the female paragenital system has an overabundance of hemocytes (Reinhardt and Siva-Jothy 2007), and bed bug hemolymph and ejaculate are suspected to contain substances or "neutralizing factors" that decrease ingested pathogen virulence and titers (Lai et al. 2016). We did not determine whether FCV was present in the bed bug hemolymph. We might speculate however that significant declines in the FCV titer over 10 days would suggest that FCV was attacked either in the digestive tract

or in the hemolymph of the bed bug, but apparently independently of the presence of *Wolbachia*.

Wolbachia has been shown to play important roles in mediating host-microbe interactions. In *Drosophila*, higher *Wolbachia* densities correlate to greater antiviral protection (Ye et al. 2017, Wong et al. 2016), and as a model for studying Blue tongue virus (BTV) replication within blood-feeding *Culicoides* midges, BTV replicated significantly in all cell lines examined from BTV-infected *Drosophila melanogaster* reared without *Wolbachia* (Shaw et al. 2012). *Wolbachia* also mediates immunocompetence in isopods (Braquart and Varnier 2008). In some mosquito-*Wolbachia*-virus interactions, *Wolbachia* primes the mosquito innate immune system (Pan et al. 2012, Kambris et al. 2010, Moreira et al. 2009), but there is evidence to show that in several mosquito examples where *Wolbachia* naturally occurs, the presence of *Wolbachia* has little to no influence on resistance to or suppression of viruses (Skelton et al. 2016, Johnson 2015b).

The localization of *Wolbachia* within the host is relevant to its involvement in pathogen suppression. In many insects, *Wolbachia* is systemically distributed either throughout the body or in specialized but highly diffuse tissues (e.g., fat body, integument). In *C. lectularius* on the other hand, *Wolbachia* resides exclusively in a bacteriome of both sexes, in association with the gonads (Hosokawa et al. 2010) and would likely not encounter the virus to initiate a symbiont-mediated immune response. While *Wolbachia* could respond to immune challenges by remotely signaling to the fat body and hemocytes, its location in the gonad-associated bacteriome would make such a signaling pathway less likely than in mosquitoes, flies and isopods.

Bed bugs do not appear to be competent vectors for most human viruses, and their status as a medically-important vector of disease remains uncertain. However, bed bugs could be more important vectors of dsDNA viruses such as hepatitis B virus (HBV). Moreover, *C. lectularius* and other cimicids conceivably may have a greater significance from a veterinary medicine perspective. Other species of cimicids may be epidemiologically important in diseases of birds and bats yet to be investigated (Adelman et al. 2013). The swallow bug *Oeciacus vicarius* can vector several arboviruses (Brown and Brown 2005), and can experimentally transmit Fort Morgan virus to uninfected birds (Rush et al. 1980). Commercial poultry operations are likely to have heavy infestations of ectoparasites (Steelman et al. 2008), and the role of bed bugs as primary or bridge vectors of avian diseases is essentially unknown. Interestingly, despite an interest over the past 100 years in the potential of bed bugs as vectors of human pathogens, including HIV, HBV, Ebola, Yellow Fever, Polio, Rabies, *Plasmodium*, *Leishmania*, *Yersinia*, and numerous bacterial species, immune responses by bed bugs when challenged with a pathogen remain poorly understood.

Conclusions

To our knowledge, this is the first study to evaluate *Wolbachia*'s influence on virus replication in *Cimex lectularius*. Our results indicate that *Wolbachia* does not play a role in ssRNA virus suppression in bed bugs, in contrast to its involvement in several other hematophagous insects. These results offer further supporting evidence that bed bugs are likely not competent vectors of ssRNA viruses, adding feline calicivirus to the list of viruses

examined thus far. Our conclusions highlight the need for future research to include (a) quantification of virus titers in various body compartments, particularly the hemolymph and salivary glands; (b) hemocoel injections of virus for titer comparison in *Wolbachia*-free and normal bed bugs; and (c) investigation of the ability of the bed bug to transmit the virus upon re-feeding.

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Table 4.1. Sex ratio in each of the three bed bug groups at sample time interval post-feeding.

Time post-feeding	Wb^+		Wb^-		Wb^- 90 d	
	M	F	M	F	M	F
5 hrs	1	2	1	2	0	3
24 hrs	1	2	2	1	1	2
4 d	2	1	2	1	2	1
7 d	2	1	1	2	2	1
10 d	3	0	2	1	2	1
Totals	9	6	8	7	7	8

Abbreviations: Wb^+ Colony bed bugs containing *Wolbachia*, Wb^- Two colonies cured of *Wolbachia* with the antibiotic rifampicin, Wb^- *90 d* Cured of *Wolbachia* with antibiotic then reared for 90 d on blood supplemented with vitamins but no antibiotic

Table 4.2. FCV titers (log_{10} $CCID_{50}$/0.1 ml) in each of the three bed bug groups at five sampling time interval post-feeding.

Treatment	Mean (± SE) FCV titers (log_{10} $CCID_{50}$/0.1 ml)				
	5 hrs	24 hrs	4 d	7 d	10 d
Wb^+	4.67 ± 0.00	4.56 ± 0.06	4.44 ± 0.06	3.06 ± 0.71	3.22 ± 0.49
Wb^-	4.89 ± 0.31	4.56 ± 0.11	5.11 ± 0.11	3.50 ± 0.50	3.78 ± 0.22
Wb^- 90 d	4.50 ± 0.40	4.45 ± 0.00	4.17 ± 0.17	3.67 ± 0.17	3.72 ± 0.15

Abbreviations: Wb^+ Colony bed bugs containing *Wolbachia*, Wb^- Two colonies cured of *Wolbachia* with the antibiotic rifampicin, Wb^- *90 d* Cured of *Wolbachia* with antibiotic then reared for 90 d on blood supplemented with vitamins but no antibiotic

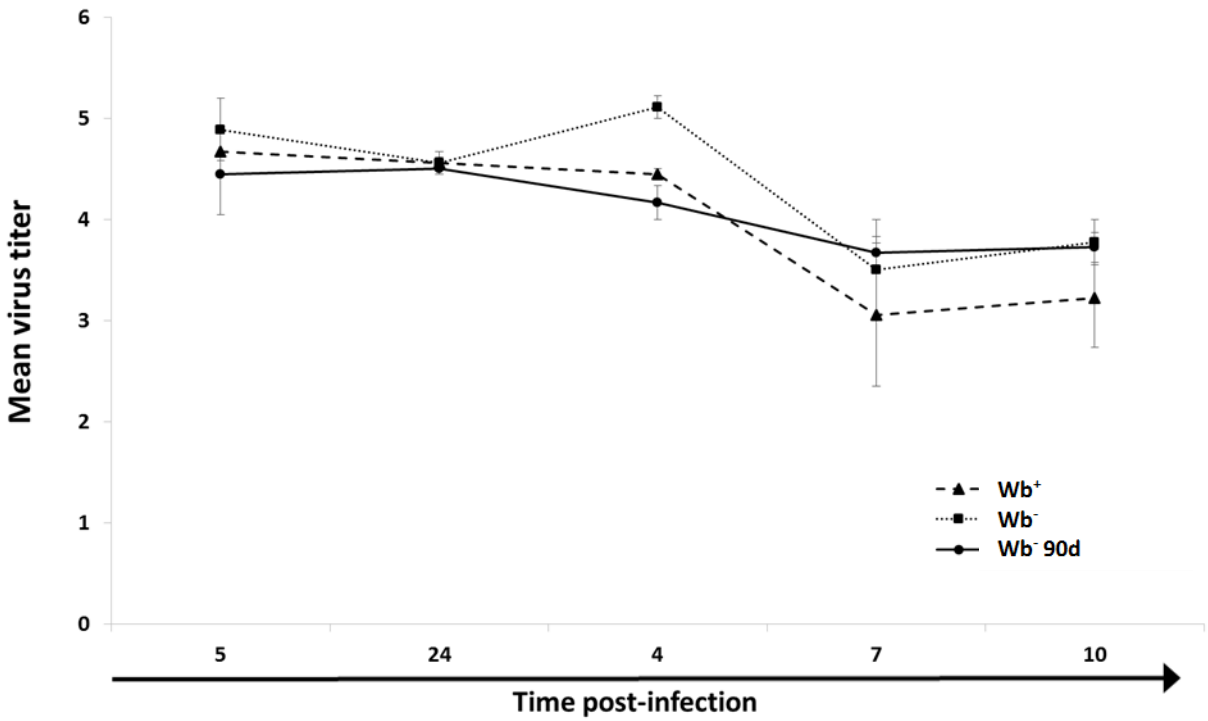

Figure 4.1. Mean (± SE) Feline calicivirus titer (log_{10} $CCID_{50}$/0.1 ml) in the three bed bug treatment groups 5 hrs to 10 d post-feeding. No cells in the cell control were infected in any treatment. Wb^+, colony bed bugs containing *Wolbachia*; Wb^-, two colonies cured of *Wolbachia* with the antibiotic rifampicin; Wb^- 90 d, cured of *Wolbachia* with antibiotic then reared for 90 d on blood supplemented with vitamins but no antibiotic.