2016

ENDOSYMBIOTIC BACTERIA IN THE BED BUG, *Cimex Lectularius* L. (HEMIPTERA: CIMICIDAE)

Mark H. Goodman
University of Kentucky, mark.goodman@uky.edu
Digital Object Identifier: https://doi.org/10.13023/ETD.2016.527

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Mark H. Goodman, Student

Dr. Kenneth F. Haynes, Major Professor

Dr. Charles W. Fox, Director of Graduate Studies

ENDOSYMBIOTIC BACTERIA IN THE BED BUG, *Cimex Lectularius* L. (HEMIPTERA: CIMICIDAE)

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture and Food Science at the University of Kentucky

By

Mark H. Goodman

Lexington, Kentucky

Director: Dr. Kenneth F. Haynes, Professor of Entomology

Lexington, Kentucky

2016

ABSTRACT OF DISSERTATION

ENDOSYMBIOTIC BACTERIA IN THE BED BUG, *Cimex lectularius* L. (HEMIPTERA: CIMICIDAE)

The return of bed bugs to prominence as an urban pest has precipitated an emphatic research response from the scientific community in an effort to better understand their biology and management. However, not all aspects of bed bug biology have received equal attention. The role of symbiotic bacteria in the normal function and biology of insects is often underappreciated, and this is true of bed bugs. Bed bugs have two main endosymbiotic bacteria, a *Wolbachia* species and a poorly-characterized gamma-proteobacterium referred to as BLS. However, their interactions with the host are poorly understood. I explored various aspects of these symbionts and their relationship with bed bugs. Major objectives included studies of the methods for symbiont elimination to create aposymbiotic strains, modes of endosymbiont transmission within a bed bug population, and impacts of symbiont loss on the host. Additional studies of bed bugs measured the impact of juvenile hormone analogs for the control of bed bugs.

Endosymbionts often form close interdependent relationships with the host, making it difficult to create a truly aposymbiotic strain. Maintaining bed bugs at elevated temperature (36°C) for five weeks or feeding for eight weeks with the antibiotic rifamycin were determined to be the most effective methods of those examined, including the use of other antibiotics. Although these two treatments were not successful in eliminating symbionts completely from treated adults, aposymbiotic strains were successfully created by harvesting the offspring of treated bugs soon after treatment.

Transovarial transmission of endosymbionts in bed bugs is well documented, but additional methods of transmission have not been explored. The reproductive strategy exhibited by bed bugs, traumatic insemination, has been suggested as a possible avenue of horizontal transmission of symbionts. My experiments demonstrated horizontal transmission of BLS from the male to the female during mating, but there was no indication that the new infection was passed from females to males or directly to offspring. No horizontal transmission of *Wolbachia* was detected. There was also no evidence of transmission through coprophagy.

In contrast with normal symbiotic controls, bed bugs which lacked both symbionts took a significantly longer time to develop to the adult stage. Successive generations of aposymbiotic bugs took longer to develop, up to several months. Supplemental B Vitamins administered through blood meals did not compensate fully for the slowed development. Upon reaching the adult stage, aposymbiotic bugs were significantly smaller than normal bugs in total body length and head-capsule width. Symbiont loss did not directly affect fecundity of adults.

Hydroprene and methoprene administered as the active ingredients of formulated products produced negative effects such as reduced fecundity and increased mortality only at doses much greater than label rate, and did not inhibit feeding or activity at sub-lethal doses. This work with Juvenile Hormone analogs has been published in *Pest Management Science.*

KEYWORDS: symbionts, *Wolbachia*, horizontal transmission, aposymbiotic

Mark H. Goodman

15 December 2016
Date

ENDOSYMBIOTIC BACTERIA IN THE BED BUG,

Cimex Lectularius L. (HEMIPTERA: CIMICIDAE)

By

Mark H. Goodman

Kenneth F. Haynes

Director of Dissertation

Charles W. Fox

Director of Graduate Studies

15 December 2016

ACKNOWLEDGMENTS

I've been blessed with much support and guidance leading up to this milestone. From the beginning of my entomological career I had encouragement from dedicated mentors. Dr. Shawn Clark, Dr. Riley Nelson, and Dr. Richard Baumann at Brigham Young University opened my eyes to the possibility of working with insects as a career, and gave me opportunities to explore entomology and conduct research even as an undergraduate.

Before I started my graduate studies I heard only good reports about Dr. Kenneth F. Haynes, and I was eager for the opportunity to work with him. He exceeded his high reputation, and has proved to be constantly supportive, kind, professional, patient yet persistent, and a genuine mentor for whom I have the greatest respect. I want to thank him for his guidance and friendship.

Dr. Michael F. Potter also played a major role in helping me to see beyond the experiments to the application of my research and professional development. He has always supported me, offering encouragement and energy when things were overwhelming.

I express gratitude to my entire graduate committee, Dr. Ken Haynes, Dr. Mike Potter, Dr. Jen White, Dr. Stephen Dobson, and Dr. Brian Stevenson for their advice and expertise, access to laboratory equipment and space, and encouragement.

Thanks to the University of Kentucky, the UK Graduate School, and the Department of Entomology for allowing me the opportunity to be a part of a great institution with such a great tradition in building successful lives. I want to acknowledge

funding support from many sources, including the University of Kentucky, Department of Entomology, Kentucky Pest Management Association, Central Life Sciences, and a National Science Foundation Graduate Student Fellowship.

When I started, our lab manager Shelby Stamper was a huge support and always went out of his way to help me. While he passed away before this dissertation was completed, his influence is still felt and remembered. Scott Bessin also kept things running in our lab and was always willing to help when I needed it.

My fellow graduate students taught me throughout my time in our lab. I want to thank Alvaro Romero, Jennifer Gordon, and Sydney Crawley especially for their friendship, collaboration, good times, and the help they often gave me. Other members of the entomology department also gave freely of their time and shared experience and resources.

It has taken me several years to complete this work, and I express love and appreciation to my wife Misty for her unfailing support in my efforts. She has remained consistently supportive of my goal despite the length of time it required and even as our family has grown. No one else has sacrificed more for my success, and I am forever indebted.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Introduction

Bed bug biology

Bed bugs are small, dorsoventrally flattened ectoparasitic insects belonging to the hemipteran family Cimicidae. There are about 110 recognized species in this family, all of which feed exclusively on blood (Usinger 1966, Henry 2009). While a majority of cimicid species are exclusively associated with bats or birds, three are associated with humans; *Leptocimex boueti* (Brumpt) is localized to West Africa, *Cimex hemipterus* (F.) (commonly called the tropical bed bug) is limited to tropical regions of the world, and *Cimex lectularius* L. (generally referred to as the bed bug) is found worldwide (Usinger 1966, Reinhardt and Siva-Jothy 2007b). *Cimex lectularius* is the predominant bed bug pest of humans in temperate regions and it is the subject of this dissertation.

Bed bugs are thigmotactic and prefer to live in protected harborages (Usinger 1966, Romero et al. 2010), often on textured surfaces such as wood or fabric. Harborages vary in size and number of individuals sheltering within; a single bug may be found in the head of a screw, or hundreds may cluster together on a mattress seam. These aggregations are composed of individual bugs that may differ in life stage, sex, and feeding or mating status (Reinhardt and Siva-Jothy 2007). Various factors such as population density, distance between harborages, presence of fecal material or pheromones, and proximity to the host impact aggregation behavior (Parashar et al. 2003, Olson et al. 2009, Pfiester et al. 2009a, Naylor 2012). There also appear to be benefits to clustering behavior in harborages, including faster development (Saenz et al. 2014) and desiccation resistance by creating microclimates of higher humidity (Benoit et al. 2007).

Bed bugs function under an endogenous circadian rhythm entrained by darkness, tending to feed and be active at night, but will adapt to activity at other times in order to take advantage of host availability (Usinger 1966, Reinhardt and Siva-Jothy 2007b, Romero et al. 2010). This behavior is similar to that of other blood-feeding Hemiptera (Barrozo et al. 2004) and these two behaviors—hiding in harborages and night activity—contribute to making bed bugs a difficult pest to eliminate.

Bed bugs have five nymphal instars, and both males and females require at least one blood meal to reach each successive developmental stage after hatching from the egg (Reinhardt and Siva-Jothy 2007). Although humans are the most common host, bed bugs can also survive and reproduce utilizing chickens, bats, and various rodents as alternative hosts (Usinger 1966, Johnson 1937).

Bed bugs have evolved an unusual form of reproduction known as traumatic insemination, with specialized paragenital organs in the female. This behavior was first described by Patton and Cragg (1913), and involves the male puncturing the ventral cuticle of the female with the curved, blade-like paramere at a particular point of thin cuticle called the ectospermalage where an internal bag-like organ called the mesospermalege has evolved to accept the injected sperm (Ribaga 1897, Rivnay 1933, Reinhardt et al. 2003). After entering the mesospermalage, sperm migrate along oxygen gradients through the hemolymph to the seminal conceptacles, and from there to the ovaries (Rao and Davis 1969, Ruknudin and Silver 1987). Traumatic insemination has negative impacts on the female, including introduction of pathogens and reduction of ability to maintain water balance (Reinhardt et al. 2005, Siva-Jothy 2006, Benoit et al. 2012). The evolutionary conflict implied in this reproductive strategy has been intensely

studied and reviewed (Stutt and Siva-Jothy 2001, Morrow and Arnqvist 2003, Pfiester et al. 2009b, Reinhardt et al. 2009, Reinhardt et al. 2015). In-depth reviews of bed bug biology, ecology, morphology, and behavior include Johnson (1941), Usinger (1966), and Reinhardt and Siva-Jothy (2007b).

Bed bugs in society

Today, bed bugs can be found in hotels, single and multi-family residences, libraries, theaters, cars and other transportation, retail spaces, hospitals, and nearly anywhere humans lie or sit long enough for feeding to occur (Robinson and Boase 2011, Potter et al. 2013, Zorrilla-Vaca et al. 2015). They may cause itchy or painful bites, anemia, emotional trauma, economic loss, and are associated with considerable social stigma and psychological ailments (Potter 2006, Pritchard and Hwang 2009, Ashcroft et al. 2015). But bed bugs have not always been considered the severe problem they currently present; while they have been associated with humans as a pest for thousands of years, historically they were more of a nuisance than a crisis (Usinger 1966, Potter 2008). The advent of central heating of homes in the early 1900's appears to have led to more serious problems with infestation, as bugs could reproduce at full capacity year-round (Potter 2008). During these years, research on bed bugs intensified, and many papers and books were published on their biology, ecology, and control. Much of that early research remains the foundation of our current knowledge and understanding about bed bugs.

With the advent of modern insecticides in the decades immediately following World War II, bed bugs quickly disappeared from developed countries and as the problem abated, much of the research focus on bed bugs also died out (Potter 2008, Reinhardt and Siva-Jothy 2007b).

The return of bed bugs to prominence as an urban pest starting in the late 1990's (Koehler 2001, Boase 2009, Potter 2008), along with their associated resistance to common insecticides (Romero et al. 2007), has precipitated an emphatic response from the scientific research community in an effort to better understand their biology and control. Our knowledge of bed bugs has expanded dramatically since the beginning of the 21st century, including new information about the roles and identity of semiochemicals in bed bug ecology (Siljander et al. 2008, Ryne et al. 2009, Harraca et al. 2010b), bed bug anatomy and physiology (Harraca et al. 2010a, Liu et al. 2014), mechanisms of insecticide resistance (Zhu et al. 2010, Romero et al. 2007, Romero et al. 2009, Gordon et al. 2014), sexual conflict and evolution of traumatic insemination (Morrow and Arnqvist 2003, Reinhardt et al. 2003, 2007a, Stutt and Siva-Jothy 2001), physiology (Otti et al. 2009, Reinhardt et al. 2009, Benoit et al. 2009), behavior (Olson et al. 2009, Pfeister et al. 2009, Cooper et al. 2015), and potential disease transmission by bed bugs (Silverman et al. 2001, Blow et al. 2001, Salazar et al. 2015).

The potential for bed bugs to transmit disease has been of real concern and the subject of repeated study for decades. The literature on this point is crowded with attempts to demonstrate the viability of pathogens in the bed bug or transmission of pathogens to vertebrates (Silverman et al. 2001, Blow et al. 2001, Goddard 2003, Goddard and deShazo 2009, Adelman et al. 2013). Recent studies have demonstrated not only the viability of *Trypanosoma cruzi* (the agent of Chagas Disease) in bed bugs, but also their potential to act as vectors of the pathogen (Salazar et al. 2015). While transmission of Chagas Disease by bed bugs has been technically shown in the lab with

mice, there is still no real-world demonstration of infectious disease transmission by bed bugs.

While there is a lot of focus on the presence of pathogenic microbes in bed bugs, a related area which is not well understood is the role of symbiotic bacteria in the normal function and biology of bed bug populations.

Symbionts in insects

The role of symbiotic bacteria in insect biology has gained more appreciation as time goes on. Since the coining of the term "symbiosis" by Anton de Bary in 1878 (Oulhen et al. 2016) to describe dissimilar organisms living together, much has been discovered and written about symbionts.

Endosymbiosis is a special case of symbiosis in which one organism lives inside another dissimilar organism (the host). These relationships occur on a continuum, ranging from mutualistic interactions in which both host and symbiont benefit from the relationship to parasitism in which the symbiont benefits to the detriment of the host (Moran et al. 2008). They can further be described as obligate, in which the host and/or endosymbiont depend on the relationship for survival, or facultative, in which the relationship is not necessary for at least one of the pair (Moran et al. 2008). Finally, within these two groups, those obligate symbionts that are required for *host* survival may be referred to as primary or P-symbionts. Facultative symbionts may be referred to as facultative mutualists when their presence is beneficial to the host. When facultative symbionts display parasitic behavior and alter host reproduction to increase their own spread, they are considered reproductive manipulators (Moran et al. 2008). Multiple

endosymbiotic organisms may exist within a single host, resulting in more complex interactions (Wu et al. 2006, Moran et al. 2008).

In the early 1900's, some of the same researchers working on bed bugs were also pioneering work on symbionts using light microscopy, histology, and experimental dissections to describe not only the appearance and location of microbes in insects, but also to explore how the microbes were transmitted (Buchner 1923, Hertig and Wolbach 1924, Hase 1930). More recently as molecular technologies, tools and techniques have become more available, there has been a surge in symbiont research and a plethora of symbiont-host interactions have been revealed. Some of these include morphological changes in the host such as color (Tsuchida et al. 2010), reproductive changes such as egg production (Dedeine et al. 2001), reduced sperm competitive ability (de Crespigny and Wedell 2006), increased mating behaviors (de Crespigny et al. 2006), or cytoplasmic incompatibility and male-killing (Bourtzis and O'Neill 1998), nutritional provisioning of B vitamins and amino acids (Nakabachi and Ishikawa 1999, Brownlie et al. 2009), defense against parasitoids (Oliver et al. 2003, Brownlie and Johnson 2009), insecticide resistance (Kikuchi et al. 2012), immune function and protection from pathogens (Scarborough et al. 2005, Haine 2007), influences on food choices (Leonardo et al. 2003, Tsuchida et al. 2004), vector competency (Beard et al. 1998, Beard et al. 2002) and so on. While many symbionts may have defined roles yet to be discovered, others may simply be commensal and have no measurable impact on the host. However it is clear that many symbionts have shown and continue to demonstrate an impact on the biology of arthropods, and bed bugs are no exception.

Symbionts in bed bugs

Anatomical studies on the unique reproductive system of bed bugs, including descriptions of some organs that contain symbionts, were published near the end of the 1800's (Ribaga 1897, Berlese 1898a, 1898b) and other similar descriptive studies followed. However, the first papers to include a description of microorganisms in bed bugs were published in 1921. Arkwright et al. (1921) used light microscopy, dissections and histology to describe two bacterial forms living inside the bed bug, emphasizing a threadlike motile form found predominantly in the Malpighian tubules, reproductive organs, and hemolymph, but also noting another minute form in the reproductive organs. They concluded that all forms were simply stages of one bacterial symbiont. In addition to describing these two bacterial forms as being different types, Buchner (1921) described an organ he called the mycetome, containing specialized cells or mycetocytes for holding endosymbionts. The mycetome is a paired organ in bed bugs that lies just above the gonads in both sexes, and is directly attached to the testes in the male. These descriptions were followed by a masterful study of bed bug development (Buchner 1923), which included descriptions of the symbionts and their movement in the bed bug embryo as it developed, going so far as to dissect the various stages of embryo before the eggs were laid. Buchner also demonstrated that both bacterial forms were transmitted transovarially. Over the next several decades, researchers continued to provide descriptions of these bacteria and their location in the host, but there was disagreement as to scientific names and how many different types were present in bed bugs (Hertig and Wolbach 1924, Dasgupta and Ray 1955, Ray and Dasgupta 1955, Dasgupta and Ray 1956, Pfeiffer 1931, Philip 1956, Krieg 1959, Chang and Musgrave 1973).

Although research on bed bug symbionts decreased as pest populations in developed nations declined, there were a few notable advances. Chang (1974) described the symbionts morphologically and attempted to eliminate them from the bed bugs by rearing them at increased temperatures of 36°C for two weeks. After treatment he noted decreased symbionts in the mycetome, which seemed to collapse. He also noted that for several weeks after the heat treatment and apparent loss of symbionts, there were no eggs laid by adult females, and suggested that there may be a symbiont role in providing nutrition. Louis et al. (1973) used scanning electron microscopy to clarify the presence of two distinct endosymbiotic bacteria, providing clear images of the motile form in particular. Despite this research, the identity and roles of bed bug endosymbionts remained unclear until new molecular technology allowed for different ways of detecting and measuring microorganisms.

In 1997, Hypsa and Aksoy were the first to sequence bacterial genes extracted from bed bugs, identifying them as a *Wolbachia* sp. in the F clade and a gamma-proteobacterium referred to as BEV-Like Symbiont (BLS). The designation BLS was based on genetic similarities of the sequenced 16S amplicon to that of a symbiont called BEV found in the sharpshooter *Euscelidius variegatus* Kirschbaum (Campbell and Purcell 1993). BLS appears to be the motile form described by Louis et al. (1973), and I was able to readily observe it in the hemolymph of bed bugs under a phase contrast microscope. The identities of the two endosymbionts have been confirmed by multiple researchers (Rasgon and Scott 2004, Sakamoto and Rasgon 2006a, Hosokawa et al. 2010, Meriweather et al. 2013), along with evidence that these two symbionts were found consistently in bed bug populations around the world (Sakamoto and Rasgon 2006b,

Sakamoto et al. 2006, Siddiqui and Raja 2015, Akhoundi et al. 2016). In the bed bug, Wolbachia is present in the mycetomes, reproductive organs, and mesospermalage; BLS is present in these same organs generally in lower concentrations, but is also found in the hemolymph and malphigian tubules (Hosokawa et al. 2010).

It is generally accepted that in order to thrive, those arthropods that feed exclusively on nutritionally deficient materials such as blood and xylem must have symbionts to provide supplemental nutrients including vitamins and amino acids (Koch 1960, Brooks 1964, Chang 1975). This has been demonstrated in aphids, tsetse flies, lice, and others (Nakabachi and Ishikawa 1999, Brownlie et al. 2009, Rio et al. 2016), leading to the suggestion that at least one of the symbionts in bed bugs must provide a nutritional benefit. In two studies, bed bugs fed on thiamine-deficient rats afterwards exhibited higher levels of thiamine than the host, suggesting that B-vitamins might be supplied to bed bugs by symbionts (De Meillon and Goldberg 1946, De Meillon et al. 1947). This host-symbiont interaction remained unproven until Hosokawa et al. (2010) published a study that demonstrated that *Wolbachia* played a major role in providing nutrients to the bed bug, without which they experienced decreased fecundity and slowed development. This was a significant finding, since most *Wolbachia* infections previously studied in other arthropod hosts had demonstrated reproductive manipulation, not benefits (Werren 1997, Werren et al. 2007, Rio et al. 2016). Perhaps the discovery of nutritional provisioning by *Wolbachia* should not be surprising, since the C, D and F clades of *Wolbachia* provide similar benefits in nematode and other arthropod hosts (Bandi et al. 2001, Nikoh et al. 2014) as well. In subsequent studies, it has been confirmed that loss of *Wolbachia* in bed bugs caused negative effects on the host such as lower fecundity on

both male and female bugs (Heaton 2013). Sequencing the genome of *Wolbachia* in bed bugs also revealed that this symbiont carries the genes necessary for producing the vitamins Riboflavin and Biotin (Nikoh et al. 2014).

Elimination of symbionts from the host

One of the key ways researchers have explored the impact of endosymbionts on a host is by removing the symbionts from part of a population, then comparing them with the control group for specific response variables. Elimination can be achieved through a few different methods depending on the specific requirements and characteristics of individual systems. For example, centrifugation has been used to eliminate louse endosymbionts by taking advantage of symbiont migration within the embryo at specific developmental time-points (Aschner 1934, Sasaki-Fukatsu et al. 2006, Perotti et al. 2007). Irradiation has been found to be effective in termites and other arthropods with thin cuticle (Novelli et al. 2008). High levels of oxygen under high barometric pressure has been effectively utilized in both cockroaches and termites, exploiting the susceptibility of their specific symbionts to oxygen toxicity at levels that had no demonstrably deleterious effect on the host (Cleveland 1925, Brooks and Richards 1955, Cleveland and Burke 1956). Some hosts are able to survive under higher temperature conditions than their symbionts, allowing the removal of symbionts by rearing at elevated temperatures (Chang 1974, Heaton 2013). When symbionts are maintained in a population by horizontal transmission from infected surfaces, as in assassin bugs, sterile rearing techniques may be effective (Eichler and Schaub 1998). The use of antibiotics can greatly simplify the process of symbiont elimination in many situations, since feeding or otherwise applying the treatment can be enough to remove all symbionts (Hermans et al.

2001). This has been the most common method for many years, documented in work with bed bugs (Hosokawa et al. 2010), aphids, mosquitos (Dobson and Rattanadechakul 2001, Xi et al. 2005), fruit flies (Brownlie et al. 2009), Tsetse flies (Pais et al. 2008), parasitoid wasps (Dedeine et al. 2001), and numerous others. In some cases, different antibiotics have even been used to target specific symbionts without harming others (Koga et al. 2007). Molecular techniques such as Denaturing Gradient Gel Electrophoresis (DGGE), PCR, real-time PCR, Elisa, and Fluorescent In-Situ Hybridization (FISH) staining may also be effective for detecting symbiont identity, presence and/or quantity, making it possible to verify the efficacy and success of these removal techniques (Higgins and Azad 1995, Fenolar et al.2003, Zouache et al. 2009).

A newly symbiont-free (aposymbiotic) group of insects becomes a more effective tool for detecting host-symbiont interactions if the new strain remains reproductively viable, because this allows for generational separation from direct treatment effects. Even when a symbiont cannot be detected through some molecular techniques, it may be present in low titers or protected from detection (Müller et al. 2013, Mee et al. 2015). Rearing out successive generations of the aposymbiotic host can demonstrate long-term loss of the symbionts and reduce confounding by treatment effects.

In bed bugs, symbionts have been successfully reduced to low levels by rearing at elevated temperatures (Chang 1974, Heaton 2013) and by feeding them on blood containing the antibiotics tetracycline or rifamycin (Hosokawa et al. 2010, Heaton 2013, Nikoh et al. 2014). But endosymbionts often form close interdependent relationships with the host, making it difficult to create a truly aposymbiotic strain lasting multiple generations.

Transmission of endosymbionts

The movement of symbionts between insect populations and generations can be categorized into two main types of transmission. Vertical transmission of symbionts from parents to offspring—particularly transovarial, through the egg from mother to offspring—is by far the most common method and can be very efficient, approaching 100% efficiency in some systems (Moran et al. 2008, Bright and Bulgheresi 2010). However, horizontal transmission between individuals and populations also has been demonstrated many times for a variety of organisms. Symbionts can be transmitted sexually (Moran and Dunbar 2006), through feeding on contaminated surfaces or coprophagy (Baines 1956), or by physical contact with infected materials (Douglas 1989).

Transovarial transmission of endosymbionts in bed bugs is well documented (Buchner 1923, Hosokawa et al. 2010), but additional methods of transmission have not been explored. The reproductive strategy exhibited by bed bugs, traumatic insemination, has been suggested as a possible avenue of horizontal transmission of symbionts (Usinger 1966). This hypothesis is supported to some degree morphologically, as the mycetome in male bed bugs is directly connected to the testes (Buchner 1923, Reinhardt and Siva-Jothy 2007b). Studies have also demonstrated that environmental microbes can be transmitted through traumatic insemination in bed bugs (Reinhardt et al. 2005). Coprophagy and transmission by way of contaminated surfaces is seen in other blood-feeding bugs in the order Hemiptera, namely assassin and kissing bugs in the genera *Rhodnius* and *Triatoma* (Baines 1956, Eichler and Schaub 1998). However, their

symbiotic bacteria are found primarily in the gut and remain viable in the feces, while bed bug symbionts are not.

Objectives

The research objectives of this dissertation have been designed to further our understanding of the bed bug, and in particular the endosymbionts associated with it.

In Chapter 2, I explore the identity and presence of symbiotic bacteria in laboratory strains of bed bugs, and determine the most effective and consistent ways to eliminate symbionts from the host while maintaining reproductive viability for further testing of aposymbiotic strains. Methods tested include various antibiotic treatments and exposure to elevated temperatures.

In Chapter 3, I test the ways in which symbionts of the bed bug may be transmitted and maintained within populations in addition to transovarial transmission. I evaluate hypotheses of horizontal transmission through traumatic insemination and coprophagy by crossing normally infected bed bugs with aposymbiotic strains, and exposing aposymbiotic neonates to feces from normal adults.

In Chapter 4, the effects of endosymbiont loss on the host are investigated with the intention of shedding light on the interactions between symbionts and host. Potential effects tested for include slowed growth and development, changes in size, nutritional deficiency, and increased mortality.

Chapter 5 details experiments on the efficacy of juvenile hormone analogs for bed bug control, using different rates of commercially available products containing the

active ingredients hydroprene and methoprene on two laboratory strains in both direct and dry residue experiments. This chapter is unrelated to the other research objectives.

Chapter 2: The elimination of endosymbionts from bed bugs, *Cimex lectularius* L. (Hemiptera: Cimicidae)

Introduction

Microbial endosymbionts of insects and their associated host interactions have been recognized and studied for many years (Buchner 1923, Hertig and Wolbach 1924, Hase 1930). Many different impacts of these associations have been described, including phenotypic influences on host coloration and size (Tsuchida et al. 2010), reproductive changes such as egg production (Dedeine et al. 2001) or cytoplasmic incompatibility and male-killing (Bourtzis and O'Neill 1998), nutritional provisioning of B vitamins and amino acids (Nakabachi and Ishikawa 1999, Brownlie et al. 2009), influences on food choices (Leonardo et al. 2003, Tsuchida et al. 2004), insecticide resistance (Kikuchi et al. 2012), immune function and protection from pathogens (Scarborough et al. 2005, Haine 2007), defense against parasitoids (Oliver et al. 2003, Brownlie and Johnson 2009), and vector competency (Beard et al. 1998, Beard et al. 2002).

In light of the wide-ranging and often unexpected roles of endosymbionts that have been described thus far, it is clear that discovering and understanding these interactions is necessary to accurately understand the biology of the host itself. For example, insect life history traits such as fecundity and development time may change dramatically depending on interactions with microbial symbionts (Harrington 1967, Pais et al. 2008).

One valuable method commonly used for assessing host-symbiont interactions is eliminating endosymbionts in a subset of the host population and subsequently comparing this secondarily symbiont-free strain (aposymbiotic) with normal control

hosts. Diverse means have been employed by researchers attempting to clear arthropods of their endosymbionts. Among these methods are the following techniques: rearing at increased temperatures in bed bugs (Chang 1974, Heaton 2013), exposure to pure oxygen at elevated barometric pressure in termites and cockroaches (Cleveland 1925; Brooks & Richards 1955), centrifugation of eggs in fleas to exploit the migration of symbionts within the embryo at specific developmental time-points (Aschner 1934, Sasaki-Fukatsu et al. 2006, Perotti et al. 2007), irradiation of the host in termites and mites (Novelli et al. 2008), sterile rearing techniques in assassin bugs (Eichler and Schaub 1998), and administration of antibiotics (Hermans et al. 2001, Koga et al. 2007). The use of antibiotics has been the most common method for many years, and is documented in work with bed bugs (Hosokawa et al. 2010), aphids, mosquitos (Dobson and Rattanadechakul 2001, Xi et al. 2005), fruit flies (Brownlie et al. 2009), Tsetse flies (Pais et al. 2008), parasitoid wasps (Dedeine et al. 2001), and numerous others.

Difficulty in creating an aposymbiotic strain is not surprising given the often complex interactions and interdependencies between host and endosymbiont. The challenge is not only to remove the symbionts, but to do so while avoiding other confounding effects on the host caused by the treatment itself and perhaps unrelated to symbiont function (Wilkinson 1998, Ballard and Melvin 2007). A newly symbiont-free (aposymbiotic) group of insects becomes a more effective tool for detecting host-symbiont interactions if the new strain is reproductively viable, because this allows for generational separation from direct treatment effects. Even when a symbiont cannot be detected through some molecular techniques, it may be present in low titers or protected from detection (Müller et al. 2013, Mee et al. 2015). Rearing out successive generations

of the aposymbiotic host can demonstrate long-term loss of the symbionts and reduce the probability of confounding treatment effects. For these types of experiments to work, one needs a consistent and effective way to remove endosymbionts from the host and create separate, reproducing strains with different symbiont profiles.

Two endosymbionts are found consistently in nearly all bed bug populations worldwide (Sakamoto and Rasgon 2006b, Sakamoto et al. 2006, Siddiqui and Raja 2015, Akhoundi et al. 2016). Both were discovered at the turn of the twentieth century and described by Arkwright et al. (1921) and others, but were first identified and placed phylogenetically by Hypsa and Aksoy (1997) as a *Wolbachia* sp. in the F clade and a gamma-proteobacterium referred to as BEV-Like Symbiont (BLS). The designation BLS was based on genetic similarities of the sequenced 16S amplicon to that of a symbiont called BEV (Bacterial symbiont of *Euscelidius variegatus*) found in the sharpshooter *Euscelidius variegatus* Kirschbaum (Campbell and Purcell 1993). The identities of these symbionts have since been confirmed by additional researchers using molecular methods (Rasgon and Scott 2004, Sakamoto and Rasgon 2006a, Hosokawa et al. 2010, Meriweather et al. 2013).

Many of the methods of symbiont elimination reported above are ineffective or incompatible with the bed bug system, and therefore were not pursued in our studies. For example, symbiont elimination by centrifugation of the eggs takes advantage of symbiont migration during the early development of the flea (Perotti et al. 2007), which does not correspond with symbiont development that occurs in the bed bug where symbionts remain in the mycetome throughout embryonic development (Buchner 1923, Usinger 1966). Sterile rearing as a method is not applicable to the bed bug system, where

transovarial transmission of the symbionts would circumvent such efforts (Usinger 1966). Cobalt-60 radiation easily penetrates the thin cuticle of mites and termites and effectively removes their symbionts (Novelli et al. 2008), but irradiation may also have other side effects on the host, such as genetic mutations which could confound treatment effects (Rao et al. 2005).

Use of hyperbaric oxygen to eliminate symbionts operates on the assumption that the microbes in question are more susceptible than the host to mortality due to high oxygen conditions (Cleveland 1925; Brooks & Richards 1955). Toxicity may be due to various factors, including inhibition of growth, interference with nutritional processes, and suppression of RNA translation due to oxygen radicals (McAllister et al. 1964, Wittner and Rosenbaum 1972). I briefly explored using this method for bed bugs in preliminary studies, qPCR conducted on bugs receiving this treatment did not indicate any decrease in symbiont levels compared with control bugs, and the method was not explored further. When subjected to high pressures of pure oxygen for less than 24 hours, bed bug mortality was low. However, when treated for longer times the mortality rate increased sharply. Treated bugs showed decreased fecundity, a response often associated with symbiont loss. However, this result can also be explained by interference in sperm movement within the female bed bugs, since oxygen gradients seem to play a major role in the migration of sperm from the mesospermalage to the seminal conceptacles (Ruknudin and Silver 1987). Sperm move toward areas of higher oxygen content, which notably include the reproductive organs (Rao and Davis 1969).

Both elevated temperature and antibiotics demonstrated encouraging preliminary results and have been used in published papers involving the endosymbionts of bed bugs

with varying degrees of success. When bed bugs were kept at 36°C for two weeks, Chang (1974) reported a reduction in the number of symbionts visible based on light microscopy and histology. He noted that the mycetome seemed to lose its integrity and decrease in size after adults were treated, and that overall fecundity was lower. However, he was unable to confirm complete elimination, and the work was inconclusive as to whether these effects were consequences of the heat treatment, the loss of symbionts, or both.

In their more recent explorations of bed bug symbionts, Hosokawa et al. (2010) fed adult bugs weekly on blood containing rifamycin at 10 µg/ml (0.001%) concentration and employed qPCR to demonstrate elimination of *Wolbachia*. They continued to administer antibiotics throughout the experiment, and did not investigate effects of aposymbiosis on subsequent generations following treatment. There was also no investigation of the role of the secondary endosymbiont, BLS, since they noted it is not present in all bed bug populations and is therefore not required for bed bug survival.

Integral to the pursuit of symbiont elimination is some means of verifying that the endosymbionts are indeed absent and not merely decreased. Early studies relied heavily on skillful dissections, light microscopy, scanning and transmission electron microscopy, and histology to detect symbiont presence (Chang et al. 1973, Louis et al. 1973). Molecular technologies such as Denaturing Gradient Gel Electrophoresis (DGGE), PCR, real-time PCR, Elisa, Fluorescent In-Situ Hybridization (FISH) staining and DNA sequencing have transformed the methodology, making it possible to verify elimination of microbes with increased confidence (Higgins and Azad 1995, Fenolar et al.2003, Zouache et al. 2009, Hosokawa et al. 2010, Heaton 2013).

My initial experiments focused first on finding the most effective methods for removal of symbionts, while additional experiments attempted to create aposymbiotic lines of bed bugs maintained over multiple generations.

Materials and methods

Bed bugs used in these experiments came from a colony (NY1) that was originally collected in Plainview, NY, in April 2007. This colony has a history of insecticide resistance to pyrethroids (Zhu et al. 2010; Gordon et al. 2014). They were maintained in the laboratory under standard conditions of 26.7° C, 65 ±5% RH, and a photoperiod of 14: 10 (L: D) h. The colony was fed weekly on a parafilm-membrane feeding system with citrated or defibrinated rabbit blood (Quad Five, Ryegate, MT) heated to 34° C by a circulating water bath (Montes et al. 2002). Virgin adult bugs were obtained by isolating recently-fed fifth instar nymphs, which subsequently molted to the adult stage. Bugs for all experiments were used 7–14 days after molting to the adult stage.

DNA extractions for experiments with Denaturing Gradient Gel Electrophoresis (DGGE), rearing under increased temperatures, or multiple antibiotics were completed with QIAGEN DNEasy Extraction Kits (QIAGEN Sciences, Germantown MD) following their recommended protocol for animal tissues. Extractions were made on the entire bed bug, which was sterilized in 70% ethanol under a sterile hood (Andrews 2013). Subsequent qPCR, if any, was completed with a MiniOpticon Real-Time PCR System (BioRad Laboratories, Inc., Hercules CA) with 20 µl reactions (9.4 µl template, 10 µl FastStart SYBR Green Master Mix (Roche Life Science, Penzberg Germany), 0.6 µl primers at 10 µM).

DGGE uses a vertical polyacrylamide gel which contains denaturing agents on a gradient to separate PCR products based on nucleotide content rather than size. For this experiment, DGGE was completed on a BioRad D-Code Universal Mutation Detection System (BioRad Laboratories, Inc., Hercules CA) following the protocols given by Andrews (2013). Bacterial DNA was amplified for DGGE analysis using 16S general primers (Andrews 2013).

Experiments evaluating the effectiveness of rifamycin were completed with DNAzol (Thermo Fisher Scientific, Inc., Waltham, MA) for extraction of DNA using a modified version of the standard protocol. For these experiments, I used an iCycler iQ Real Time PCR Detection System (BioRad Laboratories, Inc., Hercules CA) with 10 µl reactions (4.7 µl template, 5 µl FastStart SYBR Green Master Mix, 0.3 µl primers at 10 µM).

All extractions were verified and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham MA) and prepared for qPCR by dilution in pure water to produce template samples with equivalent DNA concentrations of 5 ng/µl. All qPCR followed a standard protocol (denature for 30 s at 95°C, anneal primers for 15 s at 55°C, DNA extension for 30 s at 72°C, plate read, 40 cycles) followed by a melting curve from 55°C to 95° C.

Determining levels of both BLS and *Wolbachia* with qPCR required three reactions for each sample using the appropriate primers. Primers for BLS and *Wolbachia* were designed from existing 16S sequence data on GenBank (accession numbers U65654 and AY316361.1, respectively) using the NCBI primer-designing service. The successful primers were as follows: for *Wolbachia* (859F 5'GACCCGCACAAGCGGTGGAG 3',

973R 5'AATCCGGCCGAACCGACCCT 3'), for BLS (74F 5'AGCGGCGGACGGGT-GAGTAA 3', 194R 5'GCGTGAAGCCCGAAGGTCCC 3') and a reference gene Clrpl8 (accession no. EZ419784, Clrpl8F 5'AAAGGCACGGTTACATCAAAGGTG 3', Clrpl8R 5'TAGTCTTGAACCTATAGGGGTCCC 3').

The raw fluorescence data gathered during qPCR was processed using the LinRegPCR software program (Ruijter et al. 2009). This program performs baseline corrections for each sample and uses linear regression analysis to determine PCR efficiency for each. Initial concentrations of a given amplicon in each sample, measured in arbitrary fluorescence units (N_0 values), are calculated using mean PCR efficiency per amplicon and quantitation cycle (Cq) values. The N_0 values resulting from this process were calibrated with the reference gene Clrpl8 to control for variation in the initial total template concentration, and the resulting values were $\log_2$ transformed for normality for statistical purposes. Descriptive analysis was run on these final measurements, providing a mean for titers of *Wolbachia* and BLS in control bugs. In processing data from experiments with rifamycin over generations, I set a threshold of four standard deviations below this mean, and defined "positive" as those values at or greater than the threshold, while values less than the threshold were defined as "negative". In practice, no values were close to these calculated thresholds, both positive and negative results forming separate normal distributions.

Results were analyzed with Statistix 9.0 (Analytical Software, Tallahassee FL). Mean number of eggs laid and proportion egg hatch were examined using Analysis of Variance. Significant results were analyzed using the Tukey HSD multiple comparisons,

α=0.05. Proportions were arcsine transformed before analysis. Comparisons of proportions testing positive for endosymbionts were examined using Fishers Exact Test.

Antibiotics

Tetracycline and rifamycin concentrations

A preliminary laboratory test indicated that mated female bed bugs given a single blood-meal containing 0.05% tetracycline failed to lay any eggs, in stark contrast to controls (mean 8.1 eggs, 84.1% hatch). Because *Wolbachia* is reported from bed bugs and is often associated with reproductive abnormalities (Werren et al. 2008), and because the antibiotic tetracycline is commonly used to remove *Wolbachia* from a host, I thought it possible that the dramatic loss of fecundity following antibiotic treatment was associated with the elimination of one or both symbionts. I therefore set up an experiment to test the hypotheses that antibiotic treatment with tetracycline or rifamycin decreased fecundity of bed bugs through the elimination of one or both symbionts.

The experiment consisted of eight treatments (three different concentrations of tetracycline or rifamycin, and two controls), replicated three times. Tetracycline or rifamycin were diluted with phosphate buffer saline (PBS) to three different concentrations for each antibiotic. Each PBS solution was then mixed into blood, resulting in three different final concentrations of antibiotic in blood (0.05%, 0.005%, or 0.0005%). Normal blood and blood containing PBS (10%) acted as controls.

For each treatment, 20 non-virgin adult female bed bugs were taken from the NY1 colony one week after reaching the adult stage and allowed to feed to repletion once on treatment blood. After feeding, bugs were separated into individual wells on 24-well cell culture plates (3527, Corning Inc., Corning, NY), one plate per treatment group. The

resulting fecundity was measured as eggs laid and proportion hatched. After 18 d, all bugs were fed normal blood and placed into new wells for continued measurement of fecundity. Sample adult bugs were removed at 14 and 38 d, individually extracted for whole-body DNA, and PCR was performed with universal 16S rDNA primers (Andrews 2013). The resulting bacterial profiles were analyzed by DGGE and the resulting bands sequenced (Andrews 2013). Neonate offspring resulting from each treatment and feeding were extracted for DNA and processed in like manner.

Trials with different types of antibiotics

Antibiotics have been used successfully by researchers to selectively remove specific endosymbionts from insects while leaving others at normal levels (Koga et al. 2007). I chose four antibiotics (chloramphenicol, carbenicillin, tetracycline, and rifamycin) representing different modes of action to examine their capacity for eliminating bed bug endosymbionts. Chloramphenicol is a translation inhibitor, stopping bacterial protein synthesis by reversibly binding to the 50S ribosomal subunit and blocking access by aminoacyl tRNA (Trivedi et al. 2016). Tetracycline similarly prevents translation in bacteria by binding to the 16S part of the 30S ribosomal subunit in microbes and blocking aminoacyl tRNA from binding (Trivedi et al. 2016). Carbenicillin is bacteriolytic, inhibiting the formation of peptidoglycan cross-links in the bacterial cell wall, resulting in cytolysis (Butler et al. 1970). Rifamycin binds to prokaryotic RNA polymerase, inhibiting transcription by steric occlusion (Floss and Yu 2005).

In preliminary tests to determine palatability of different antibiotic concentrations in blood, each of these antibiotics was diluted with PBS into blood with three different final concentrations (0.05%, 0.005%, or 0.0005%) using the methods described above.

Because chloramphenicol was initially dissolved in 100% ethanol, an additional control was used containing PBS with ethanol in corresponding concentrations (8%, 0.8%, or 0.08% ethanol in blood).

After feeding, all bugs within a treatment group were allowed to mate for three d, then separated into individual wells on a 24-well cell culture plate. The resulting fecundity was measured as eggs laid and proportion hatched. After one week, all bugs were again fed treated blood and placed into new wells for continued measurement of fecundity. This was repeated for a third week.

Bed bugs readily fed to repletion when presented with blood containing rifamycin or carbenicillin at the highest concentration (0.05%) and fecundity remained high after multiple weeks of feeding, so this rate was used for continued experiments with these two antibiotics. At that same concentration, however, bugs would not feed on blood containing chloramphenicol. This was probably due to the high alcohol content (8% of blood) used in the chloramphenicol treatment, as bugs also would not feed on the ethanol control at that level. While tetracycline was readily accepted by bugs at the highest dose, it resulted in significant mortality and complete loss of fecundity. Chloramphenicol and tetracycline were therefore used at the intermediate level (0.005%) for continuing experiments.

Following this initial testing, I examined the effect of these different antibiotics on symbiont titers using the antibiotic concentrations determined above (chloramphenicol 0.005%, rifamycin 0.05%, tetracycline 0.005%, carbenicillin 0.05%). Initially, virgin adult male and female bed bugs were placed in groups of 60 female and 30 male for each treatment and fed their respective treatments. All bugs within each treatment were then

placed together for 48 hr in a petri dish and allowed to mate, after which they were separated into individual wells on a 24-well cell culture plate and subsequent fecundity was measured for each female as eggs laid and proportion hatched. Seven days after the first feeding, all the bugs were allowed to feed again on their respective treatment, then placed in new wells on cell culture plates for fecundity measurements. The process was repeated through eight feedings, after which samples (adults and offspring) were taken from each treatment for extraction and qPCR testing of symbiont levels. The experiment was replicated 3 times.

Rifamycin with generations

Based on the results of the experiments with different antibiotics, a similar protocol was used, this time with only two treatments and intended to test for the long-term loss of symbionts. Virgin adult male and female bed bugs were fed weekly for eight weeks on blood containing rifamycin (0.05% final concentration), with control bugs feeding on blood containing PBS. A sub-sample of bugs were removed from each treatment after the first and all subsequent feedings, individually extracted for total-body DNA, and qPCR was used to determine the titers of both endosymbionts relative to control bugs.

After eight weeks of treatment, the bugs were paired and allowed to mate within treatments. The resulting offspring (F1) first instar nymphs were collected and reared on normal blood with weekly feedings until they reached the adult stage. At that point, samples were taken for qPCR, and the new F1 adults were allowed to mate. Offspring (F2) were again collected and reared to adulthood, and this process was repeated for the F3 generation. For each replicate, I attempted to rear and test at least three generations

from the treated bugs in order to demonstrate real and permanent loss of symbionts in the strain.

Maintenance at elevated temperatures

Initial experiments following the methods of Chang (1974) with bugs maintained at 36°C for two weeks resulted in reduced levels of endosymbionts but not complete elimination, as indicated through qPCR in comparison with controls. The reduced symbiont titers rebounded back to normal levels in the weeks following treatment. Based on these results, I maintained treatment groups at 36°C for a longer treatment time of five weeks in my experiments.

Virgin adult male and female bed bugs were maintained at either 36°C or 24°C in one of two incubators for five weeks, with four males and four females removed each wk for DNA extraction and qPCR to measure symbiont titers. The assignment of incubator to elevated temperature or control treatments was rotated over the four replicates to control for incubator effects. After five weeks the bugs were allowed to mate within the treatment and control groups. The resulting offspring (F1) neonates were collected and maintained at normal rearing temperatures. They received weekly feedings until reaching the adult stage, at which point samples were taken for qPCR and mating was allowed within each treatment group. Resulting offspring (F2) were again collected and reared. This rearing cycle was repeated for over a year, with varying number of generations dependent on development rate. I attempted to maintain each group for at least three generations, with adults being harvested at each of the first three generations (F1, F2, F3) to assess the permanence of any apparent symbiont loss.

Results

Antibiotics

Tetracycline and rifamycin concentrations

There was a significant treatment effect on mean number of eggs laid per female ($F(7,16)=25.02$, $p<0.01$), as well as the proportion of those eggs hatching ($F(7,15)=2.88$, $p=0.04$). The mean number of eggs laid per female fed on the highest dose of tetracycline (0.05, SD=0.06) was reduced relative to the untreated control (7.4, SD=0.46). No other treatments were significantly different from the control in the number of eggs laid (Table 2.1). There were two groups of treatments within which the mean proportions of eggs hatching were not statistically different. Only the treatment given the highest dose of tetracycline (0.42, SD=0.42) was significantly different from the controls (0.95, SD=0.02) in the proportion of eggs hatched (Table 2.1). The fecundity of bugs that were fed rifamycin was not significantly different from the control.

The DGGE analysis revealed that bed bugs treated with tetracycline still had detectable levels of both symbionts, even at the highest dose (Figure 2.1, H and K). The offspring of these treated bugs still had both symbionts present. However, bugs that were treated with rifamycin and their resulting progeny no longer had detectable levels of BLS. *Wolbachia* was still detected in adults and their offspring after treatment with a single dose of either antibiotic.

Trials with different types of antibiotics

There was a significant effect of treatment on levels of BLS ($F(4,24)=6.1$, $p<0.01$) and *Wolbachia* ($F(4,24)=8.96$, $p<0.01$). Tetracycline and rifamycin treatments were the most effective at removing either endosymbiont over eight weekly feedings (Figure 2.2).

Based on qPCR results, only these two antibiotic treatments resulted in symbiont levels significantly different than the PBS control. In addition, offspring of the bugs treated with these two antibiotics exhibited similarly low levels (Figure 2.2).

However, there appeared to be notable side effects of tetracycline including high mortality, few eggs being laid, and none hatching (Table 2.2). There was a significant effect of treatment on mean number of eggs laid ($F(5,12)=43.57$, $p<0.01$) and the proportion of eggs that hatched ($F(5,12)=97.54$, $p<0.01$). Based on these studies and the need for aposymbiotic lines that could reproduce effectively, rifamycin was selected for use in follow-up symbiont removal experiments.

Rifamycin with generations

The proportions of bed bugs testing positive for symbionts were significantly lower for those fed on blood containing rifamycin than for the control bugs for all eight weeks (Fig 2.3). In control samples, the proportions of bugs that tested positive after each of the eight weeks of treatment were all close to 1. However, the positive proportions changed over time for bugs fed rifamycin. Proportions of treated bugs positive for BLS dropped for the first two weeks, then rose slightly to about 0.4 and remained at that level for the final five weeks. Proportions testing positive for *Wolbachia* fell dramatically to zero after the first two weeks and stayed near zero for the remainder of the treatments. In both cases, there was no notable change in the proportion of bugs with symbionts after the third week.

No offspring of bugs from treatment groups in replicates 1 and 2 tested positive for symbionts using qPCR. For the third replicate, there were four specimens that tested positive for both symbionts in F1, but none were positive in F2. In control bugs the

proportions testing positive for BLS in F1 and F2 were 0.78 (n=18) and 0.9 (n=10) respectively. Repeated measurements of the levels of BLS in the main NY1 colony resulted in a mean proportion of 0.91 testing positive, which is not significantly different from the proportion measured in the controls (p=0.0871, p=1, Fisher's Exact Test, α=0.05). Every control bug tested positive for *Wolbachia* in the F1 and F2 generations.

Maintenance at elevated temperatures

Bugs maintained at 36°C for five weeks showed high variance in the measured titers of symbionts by qPCR, even within replicates (Figure 2.4). As a result there was no significant reduction in symbiont titers even though measured titers were lower than the control group for all weeks of treatment as well as the resulting offspring. Of the four replicates, only one resulted in an aposymbiotic strain over multiple generations.

Discussion

Efficacy of different antibiotics

Antibiotics are the most commonly used method of endosymbiont removal (Moran et al. 2008), and tetracycline is one of the more commonly used over the last decades. In light of this, the negative impact of tetracycline on fecundity and the increased mortality associated with treatment in my studies—which do not seem to be associated directly with symbiont loss—are quite surprising. Bugs treated with tetracycline experienced these effects even after one feeding, when they still had high levels of both symbionts. Potential complications with tetracycline toxicity could be associated with binding to blood serum such as would take place in blood fed to bed bugs (Rolinson and Sutherland 1965) and toxic breakdown products (Benitz and Diermeier 1964). Tetracyclines have also recently been shown to impair the function of

mitochondria in eukaryotes, with potentially confounding influence on research looking at the effects of symbiont loss. (Ballard and Melvin 2007, Moullan et al. 2015, Chatzispyrou et al. 2015).

There were no similar effects observed in bugs treated with rifamycin or the other antibiotics, all of which had normal fecundity and low mortality even after eight weeks of treatment. Only rifamycin was effective, in stark contrast with tetracycline; bugs treated with rifamycin had normal fecundity and low mortality despite having little to no detectable levels of symbionts following treatment. Thus, among the antibiotic types tested in this dissertation rifamycin is the most effective choice for curing bed bugs of their symbionts. Other antibiotics in the ansamycin family would likely have comparable results (Floss and Yu 2005), since with the exception of tetracycline these results with different antibiotics are consistent with other recent studies. For example, Fenolar et al. (2003) tested 13 antibiotics against *Wolbachia* from mosquitoes and found that antibiotics in the tetracycline and rifamycin groups were the most effective at reducing the bacterial titers, while Hermans et al. (2001) tested five antibiotics and also found that the same families of antibiotics was the most effective.

It appears from my results that the loss of both symbionts in a bed bug does not negatively impact individual fecundity over the short term. This does not necessarily mean that there are no immediate impacts, but none were apparent in my study. These results are not consistent with other recent studies using similar methodologies with bed bugs, in which immediate negative impacts on fecundity of treated individuals, such as low egg viability, were displayed (Hosokawa et al. 2010, Nikoh et al. 2014). One notable difference between the studies is their use of bed bug strains that only contained

Wolbachia, while my studies were completed on a strain that originally had both *Wolbachia* and BLS. In the generational study with rifamycin, a proportion of the treated adults continued to show detectable levels of BLS throughout the treatment period, but *Wolbachia* levels were not detected after three treatments. Perhaps this indicates some resistance in BLS to the antibiotic or its lack of penetration to all symbiont locations within the host.

While the use of different strains (with and without BLS) may not explain the different results, it points to the possibility of BLS as an additional nutritional symbiont. An overlap in provisioning or complementarity is not unreasonable, and has been demonstrated in other insects such as the sharpshooter *Euscelidius variegatus* (Wu et al. 2006, Moran et al. 2008).

Since levels of bacteria remained constant after three weeks of treatment, there may be no real benefit in treating bugs with antibiotics for a longer period. It was only by collecting offspring of treated bugs that I was able to successfully grow a BLS aposymbiotic strain that no longer required antibiotic treatment to maintain.

Efficacy of heat treatments

While maintaining bugs at increased temperatures shows some promise for removal of symbionts, the inconsistency among replicates made it ineffective for these studies. Further studies could be done to refine the process and perhaps make it more reliable. Heaton (2013) used elevated temperature extensively in her work on *Wolbachia* in bed bugs, but dealt with similar concerns of incomplete elimination of the symbiont. These concerns over rebounding symbiont titers restricted the interpretations of experimental results. On the other hand, Guruprasad et al. (2011) noted differences in

fecundity between female tachinid flies when symbionts were removed by antibiotics versus elevated temperature. They suggested that there were direct effects of the antibiotic on the host unassociated with symbiont loss, and determined that temperature-based removal of symbionts was more effective. While other researchers report good success with symbiont elimination by elevated temperatures in various arthropods including flies, nematodes and mites (Van Opijnen and Breeuwer 1999, Guruprasad et al. 2011), based on our results the most efficient and reliable method of symbiont removal for bed bugs seems to be feeding virgin adults with rifamycin for multiple weeks, allowing them to mate with other virgin treated adults, and using the resulting offspring to create aposymbiotic strains.

Table 2.1. Fecundity results following a single feeding on blood containing various concentrations of tetracycline and rifamycin. Asterisk indicates statistically significant difference from the control (Tukey HSD all-pairwise comparisons, α=0.05, n=3).

Treatment	**Mean No. Eggs per female** ± **SEM**	**Proportion hatched** ± **SEM**
Control	8.55 ± 0.99	0.92 ± 0.04
PBS control	7.40 ± 0.26	0.95 ± 0.01
Tetracycline 0.05%	0.05 ± 0.04 *	0.42 ± 0.42 *
Tetracycline 0.005%	6.97 ± 0.12	0.88 ± 0.04
Tetracycline 0.0005%	7.53 ± 0.54	0.95 ± 0.01
Rifamycin 0.05%	6.98 ± 0.66	0.94 ± 0.02
Rifamycin 0.005%	7.78 ± 0.63	0.94 ± 0.02
Rifamycin 0.0005%	7.63 ± 0.37	0.96 ± 0.01

Table 2.2. Fecundity results following 8 weekly feedings on blood containing various concentrations of four different antibiotics. Asterisk indicates statistically significant difference from the control (Tukey HSD all-pairwise comparisons, α=0.05, n=3).

Treatment	**Mean No. Eggs per female ± SEM**	**Proportion hatched ± SEM**
PBS Control (n=90)	8.963 ± 0.211	0.881 ± 0.023
Tetracycline 0.005% (n=74)	0.131 ± 0.022 *	0.000 ± 0.000 *
Rifamycin 0.05% (n=100)	7.786 ± 0.923	0.887 ± 0.010
Carbenicillin 0.05% (n=96)	9.964 ± 0.768	0.704 ± 0.105 *
Chloramphenicol 0.005% (n=104)	8.327 ± 0.346	0.921 ± 0.011
Ethanol Control (n=94)	7.194 ± 0.330	0.934 ± 0.016

Figure 2.1. Results of DGGE showing symbiont presence or absence with antibiotic treatments at 0.05%. **A:** Negative control. **B:** Positive control. **C:** Nymphs from second feeding of rifamycin adults. **D:** Nymphs from second feeding of control adults. **E:** Nymphs from first feeding of tetracycline adults. **F:** Nymphs from first feeding of rifamycin adults. **G:** Nymphs from first feeding of control adults. **H:** Tetracycline adult after 38 days. **I:** Rifamycin adult after 38 days. **J:** Control adult after 38 Days. **K:** Tetracycline adult after 2 weeks. **L:** Rifamycin adult after 2 weeks. **M:** Control adult after 2 weeks. **N:** *Cimex lectularius* (18S). **O:** *Wolbachia*. **P:** BLS.

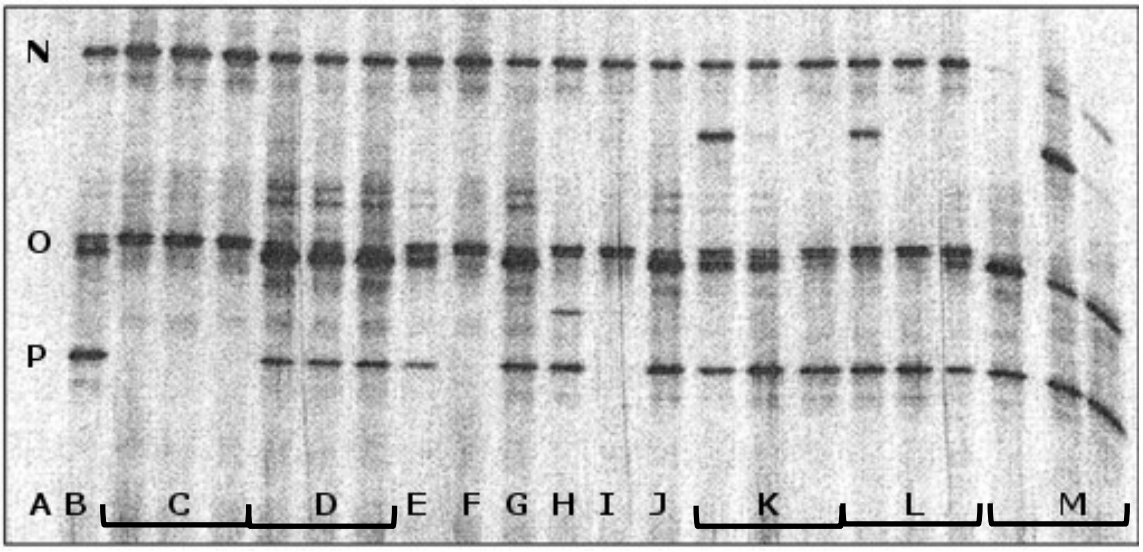


Figure 2.2. Levels of symbionts present in bed bugs after feeding on one of four antibiotics for eight weeks. Treatments include carbenicillin, chloramphenicol, phosphate buffer saline (control), rifamycin, and tetracycline. Three bars are given for each treatment, corresponding with adult female and male bugs and the resulting offspring. Bars show standard error, n=3. There were no offspring after treatment with tetracycline, therefore no measurement is given.

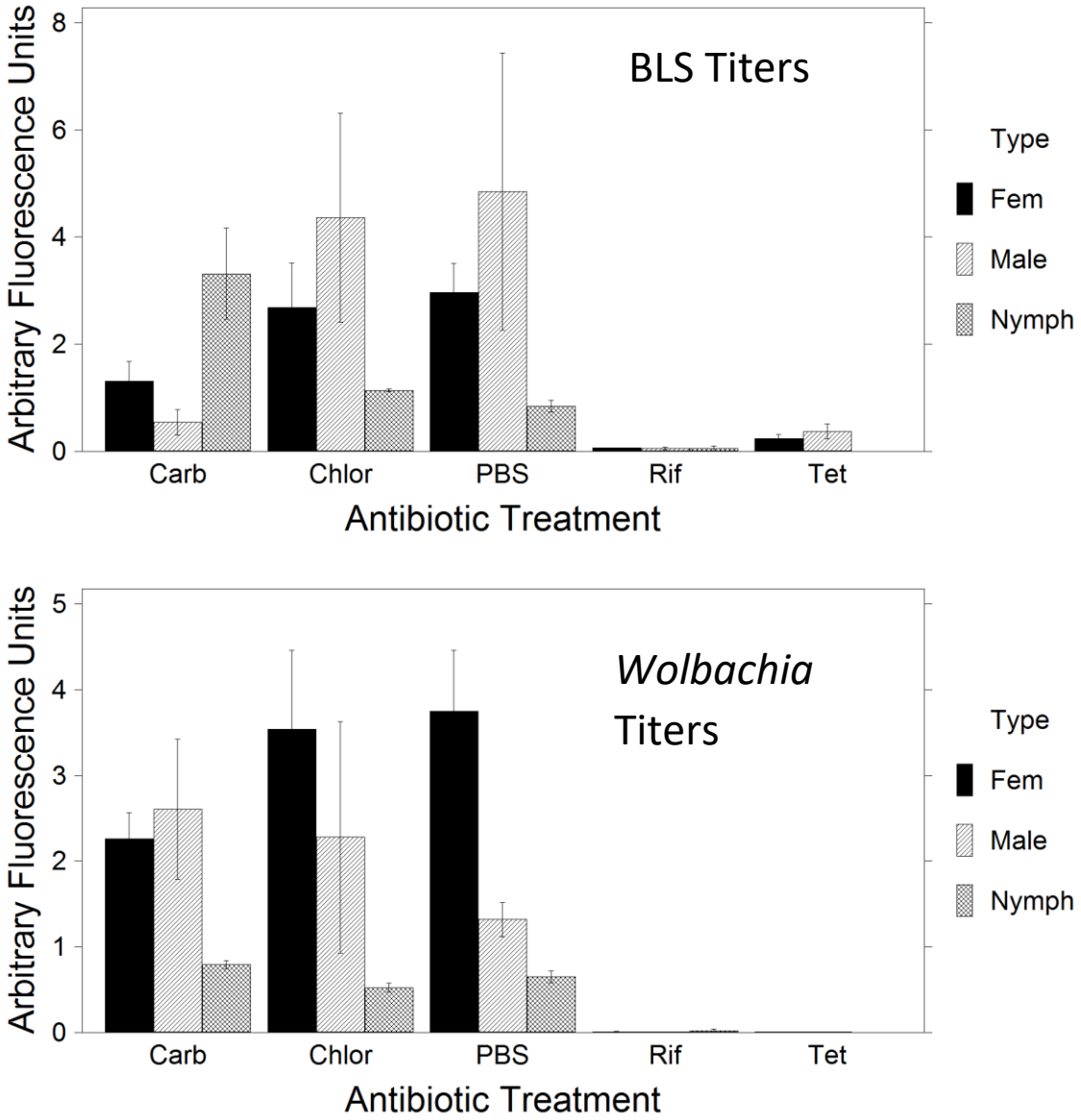
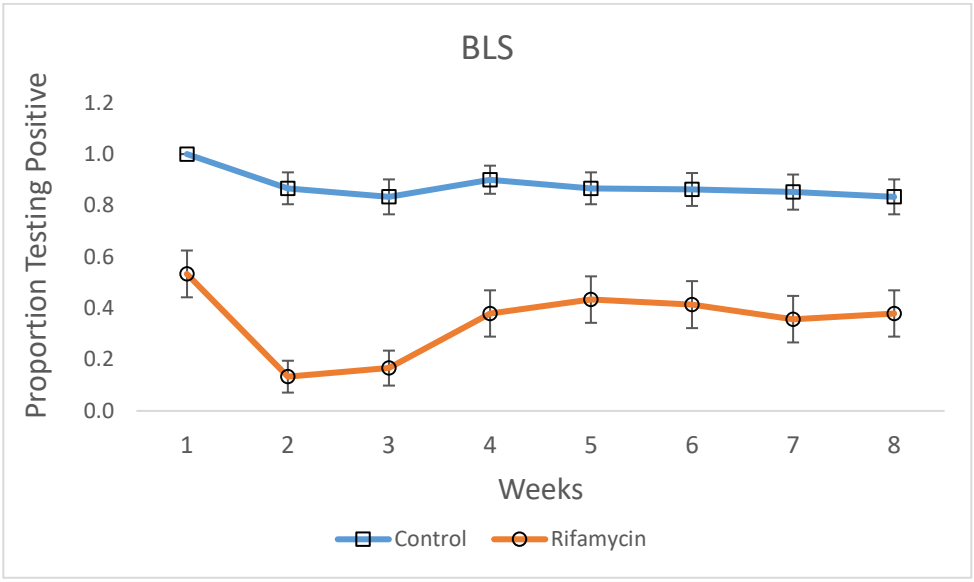


Figure 2.3. The proportion of bugs testing positive for endosymbionts over an eight week period of weekly feeding. Bugs were fed on blood with rifamycin or control blood with phosphate buffer saline. Bars show standard error of the mean.



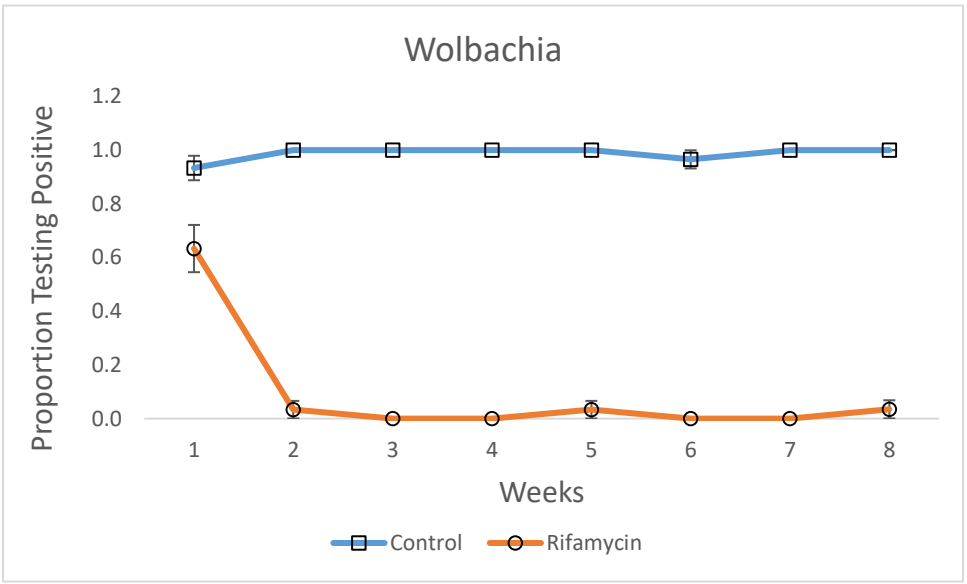
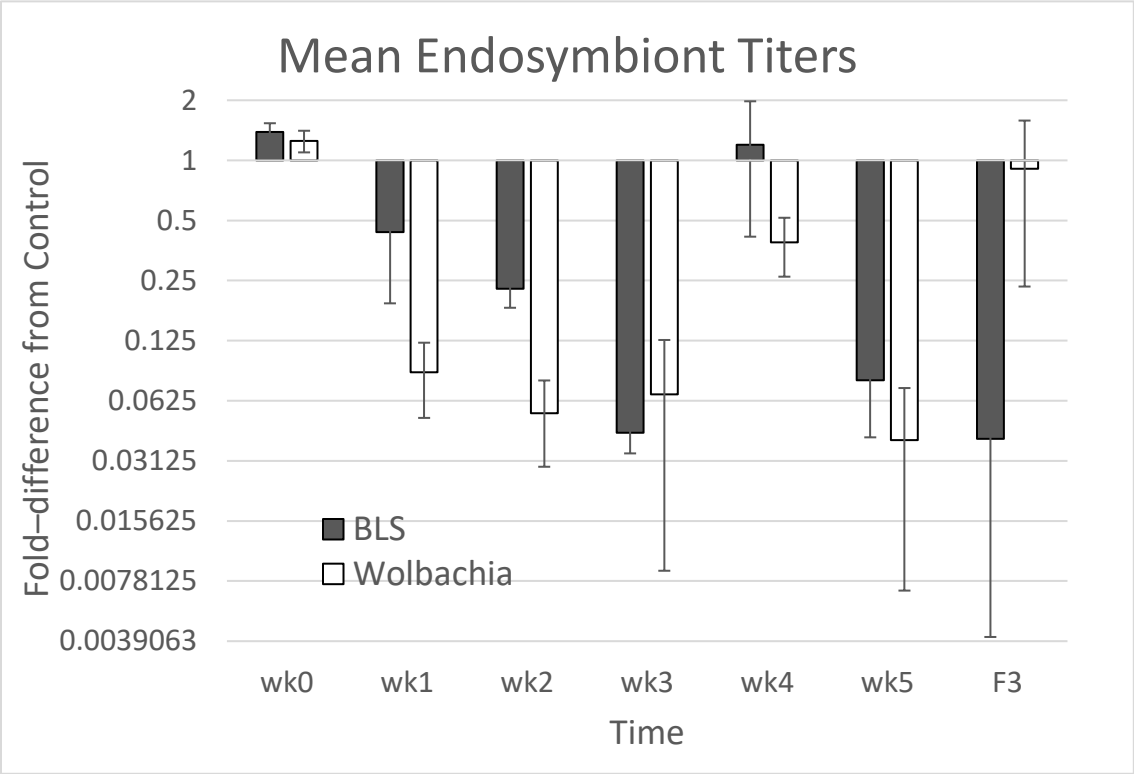


Figure 2.4. Symbiont titers in bugs maintained at 36°C for five weeks. Results are for each week of treatment and the F3 generation following treatment. Y-axis shows the fold difference (Log_2) of endosymbiont titers compared to control, with a fold difference of 1 indicating no difference from control. Bars show standard error of the mean.



Chapter 3: The horizontal transmission of endosymbionts in the bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae)

Introduction

Endosymbiotic microorganisms are found in many insects, with a variety of types and host-symbiont interactions having been described (Moran et al. 2008). Two endosymbionts are found consistently in bed bug populations around the world (Sakamoto and Rasgon 2006b, Sakamoto et al. 2006, Siddiqui and Raja 2015, Akhoundi et al. 2016). These were discovered at the beginning of the twentieth century by Arkwright et al. (1921) and others, but first identified and placed phylogenetically by Hypsa and Aksoy (1997) as a *Wolbachia* sp. in the F clade and a gamma-proteobacterium referred to as BEV-Like Symbiont (BLS) based on similarities to a symbiont found in sharpshooters (Campbell and Purcell 1993). The identities of these symbionts have since been confirmed by multiple researchers using molecular methods (Rasgon and Scott 2004, Sakamoto and Rasgon 2006a, Hosokawa et al. 2010, Meriweather et al. 2013). In 1923, Buchner published a seminal paper on the biological development of the bed bug, including a detailed description of transovarial transmission of the endosymbionts from the female to her offspring. Since that time, the vertical transmission he detailed has been consistently and universally reported as the way in which the symbionts are transferred (Usinger 1966, Reinhardt and Siva-Jothy 2007b, Hosokawa et al. 2010).

However, there are notable instances of alternative transmission methods being utilized in other insects, even within the order Hemiptera. A prime example of this is assassin bugs of the genera *Triatoma* and *Rhodnius*. These true bugs are also obligate blood feeders, but their endosymbionts are maintained through coprophagy and transfer

from contaminated surfaces rather than through transovarial transmission (Baines 1956, Douglas 1989, Eichler and Schaub 1998). Nymphs ingest wet fecal material of both parents and other adults, from which they acquire the microbial symbionts, resulting in symbiont transfer that may be both horizontal and vertical (Brecher and Wigglesworth 1944).

Despite some similarities between the hosts, coprophagy is an unlikely prospect for endosymbiont transmission in bed bugs because the symbionts of interest have not been isolated from the bed bug gut. There are no modern reports of fecal feeding by bed bugs of any stage, and despite multiple attempts with hundreds of bugs, I did not observe any fecal feeding by starved nymphs placed with recently fed adults. In an un-replicated experiment I extracted DNA from fecal material of infected bugs and analyzed for symbiont DNA using qPCR and 16S primers. There was no symbiont DNA detected in the feces, although some host DNA was recorded.

Researchers have also demonstrated horizontal transfer of symbionts through mating in aphids (Moran and Dunbar 2006) and in a parasitic wasp with *Wolbachia* (Huigens et al. 2004). However, neither coprophagy nor mating have been seriously examined as an additional mode of endosymbiont transmission in bed bugs.

Historically, there has been some speculation about bed bugs with regards to a link between the male reproductive system and transmission of symbionts to their offspring. Usinger (1966) made mention of this in his monograph, noting that the mycetome housing the endosymbionts is directly connected to the testis in the male. Bed bugs employ a form of reproductive behavior that, while not unique to bed bugs, is certainly not common among insects (Stutt and Siva-Jothy 2001, Reinhardt et al. 2009,

Tatarnic et al. 2014). Traumatic insemination in bed bugs entails the male using its blade-like paramere to puncture the female integument at a particular point of cuticular weakness, called the ectospermalage (Patton and Cragg 1913, Usinger 1966, Morrow and Arnqvist 2003). Ejaculate is received into a specialized internal bag-like structure called the mesospermalage, located just inside the cuticle at that area (Ribaga 1897, Rivnay 1933, Reinhardt et al. 2003). The mesospermalage is a loose organization of cells thought to be involved in immune function and protection from introduced pathogens during mating (Reinhardt et al. 2005, Siva-Jothy 2006). From there the sperm migrate through the hemolymph to the primary reproductive system, following oxygen gradients into the seminal conceptacles and to the ovarioles (Rao and Davis 1969, Ruknudin and Silver 1987), where fertilization occurs just before chorion development (Usinger 1966). The evolutionary conflict associated with this reproductive strategy has been well studied and reviewed (Stutt and Siva-Jothy 2001, Morrow and Arnqvist 2003, Pfiester et al. 2009b, Reinhardt et al. 2009, Tatarnic et al. 2014, Reinhardt et al. 2015).

As an endosymbiont, BLS is motile and can be readily found in the hemolymph as well as gonads in infected individuals (Louis et al. 1973, Hosokawa et al. 2010), which suggests that if it were introduced into the female through mating, it might move with the sperm and establish in the new host. *Wolbachia* is also present in both the testes of the male and the mesospermalage of the female (Hosokawa et al. 2010). It has been shown that other environmental microbes may be transferred to the female by the contaminated male during traumatic insemination (Reinhardt et al. 2005), but similar transmission between individual bed bugs has not been looked at with endosymbionts.

In light of these results, and given the aforementioned association of the mycetome with the testis in male bed bugs, my focus was turned to determining if traumatic insemination in bed bugs acted as a means of transmitting symbionts between hosts. I set up reciprocal crosses with normal and aposymbiotic bed bugs and measured the levels of symbionts present in the experimental bugs and their offspring after four weeks of mating with weekly feeding.

Materials and methods

Bed bugs used in these experiments came from a colony (NY1) that was originally collected in Plainview, NY, in April 2007. This colony has a history of insecticide resistance to pyrethroids (Zhu et al. 2010; Gordon et al. 2014). They were maintained in the laboratory under standard conditions of 26.7° C, 65±5% RH, and a photoperiod of 14: 10 (L: D) h. The colony was fed weekly on a parafilm-membrane feeding system with defibrinated rabbit blood (Quad Five, Ryegate, MT) heated to 34° C by a circulating water bath (Montes et al. 2002). Virgin adult bugs were obtained by isolating recently-fed fifth instar nymphs. Bugs for all experiments were used 7–14 d after molting to the adult stage.

DNA extractions for the crossing experiment with bugs maintained at elevated temperatures were completed with DNAzol (Thermo Fisher Scientific, Inc., Waltham, MA) using a modified version of the standard protocol. The protocol for successful extraction required close adherence to specific requirements. First, place the sample to be extracted into a microcentrifuge tube with 100µl of DNAzol. Homogenize, then let stand for 15 min. Centrifuge at 10,000 g for 10 min, then remove supernatant to a new tube with 45µl of 100% ethanol and mix thoroughly. Let stand for 3 min, then centrifuge at

16,000 g for 15 min. As a wash to remove contaminants, discard supernatant and pipette 160 µl of 75% ethanol into the tube. Mix well, then centrifuge for 5 min at 16,000 g. Repeat with a second wash and centrifuge, then remove and discard supernatant. Finally, place 50 microliters of PCR-quality water into each tube and mix well. Keep extracted DNA frozen until use.

The success of all extractions were verified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham MA) to measure concentrations of DNA and monitor quality. Each was then diluted in pure water to produce template samples with equivalent DNA concentrations of 5 ng/µl.

Determining levels of both BLS and *Wolbachia* with qPCR required three reactions for each sample using the appropriate primers. Primers for BLS and *Wolbachia* were designed from existing 16S sequence data on GenBank (accession nos. U65654 and AY316361.1, respectively) using the NCBI primer-designing service. The successful primers were for *Wolbachia* (859F 5'GACCCGCACAAGCGGTGGAG 3', 973R 5'AATCCGGCCGAACCGACCCT 3'), BLS (74F 5'AGCGGCGGACGGGTGAGTAA 3', 194R 5'GCGTGAAGCCCGAAGGTCCC 3') and a reference gene (accession no. EZ419784, Clrpl8F 5'AAAGGCACGGTTACATCAAAGGTG 3', Clrpl8R 5'TAGTCTTGAACCTATAGGGGTCCC 3',).

Two different qPCR systems were used, one for each experiment. For the crosses with bugs maintained at elevated temperatures I used a MiniOpticon Real-Time PCR System (BioRad Laboratories, Inc., Hercules CA) with 20 µl reactions (9.4 µl template, 10 µl FastStart SYBR Green Master Mix (Roche Life Science, Penzberg Germany), 0.6 µl primers). All qPCR followed a standard protocol (denature for 30 s at 95°C, anneal

primers for 15 s at 55°C, DNA extension for 30 s at 72°C, plate read, 40 cycles) followed by a melting curve from 55° to 95° C.

For the crosses with aposymbiotic strains I used an iCycler iQ Real Time PCR Detection System (BioRad Laboratories, Inc., Hercules CA) with 10 µl reactions (4.7 µl template, 5 µl FastStart SYBR Green Master Mix, 0.3 µl primers) and the same protocol as that used in the other qPCR system.

The raw fluorescence data gathered during qPCR was processed using the LinRegPCR software program (Ruijter et al. 2009), which performs baseline corrections for each sample and uses linear regression analysis to determine PCR efficiency for each. Mean PCR efficiency per amplicon and quantitation cycle (Cq) values are used to calculate the initial concentrations of a given amplicon in each sample, measured in arbitrary fluorescence units (N_0 values). After processing with LinRegPCR, the resulting N_0 values were calibrated with the reference gene Clrpl8 to control for variation in the initial template concentration, and the resulting values were $\log_2$ transformed for normality. Descriptive analysis was run on these final measurements, providing a mean for titers of *Wolbachia* and BLS in control bugs. I set a threshold of four standard deviations below this mean, and defined "positive" as those values at or greater than the threshold, while values less than the threshold were defined as "negative". In practice, no values were close to these thresholds, both positive and negative results forming separate normal distributions.

Results were analyzed with Statistix 9.0 (Analytical Software, Tallahassee FL). Mean number of eggs laid and proportion egg hatch were examined using Analysis of Variance. Proportions were arcsine transformed before analysis. Comparisons of proportions testing positive for endosymbionts were examined using Fishers Exact Test.

Crosses with individuals maintained at elevated temperatures

I placed virgin male and female bed bugs into cylindrical plastic containers used for normal colony rearing (42422, Consolidated Plastics, Stow, OH, radius 2.5 cm, depth 5 cm) as groups of 200 males or females. Groups were randomly assigned to be maintained at temperatures of 36°C or 24°C for four weeks with weekly feeding according to laboratory protocols. Previous experiments demonstrated that maintaining bugs at 36°C for this length of time reduced symbiont titers to levels not detected by our qPCR. At the end of the treatment period, four treatment groups were created by placing treated and control bugs together in pairs (40 pairs per group) into individual wells on 24-well cell culture plates (3527, Corning Inc., Corning, NY) to mate. Treatment 1, the positive control, contained normal males and females. Treatment 2 contained normal females and treated males, while Treatment 3 contained treated females and control males. Treatment 4, the negative control, contained treated males and females.

Every pair within each group was allowed to mate throughout the experiment. Fecundity was recorded for each, measured as the number of eggs laid and the proportion hatched. After two weeks, the bugs were all fed again, and the resulting fecundity was measured. Four weeks after the pairs were placed together, 10 pairs were taken from each treatment for whole-body extraction of DNA and processing with qPCR to determine final symbiont titers.

Crosses with bugs from symbiont-free strains

In order to investigate the transmission of endosymbionts in bugs which had not been directly treated to remove symbionts, I used three aposymbiotic strains of bugs, the progeny of NY1 adults directly treated with heat and the antibiotic rifamycin. Virgin adults were obtained from these three strains, and each strain served as a replicate. In addition, virgin adult bugs were obtained from three control populations ("normal" bugs) taken from NY1 at the same time the aposymbiotic groups were established. Using these sources, four treatment groups were created by placing aposymbiotic and normal bugs together as mating pairs into individual wells on 24-well cell culture plates. Treatment 1, the positive control, consisted of normal males and females. Treatment 2 consisted of normal females and aposymbiotic males, while Treatment 3 consisted of aposymbiotic females and control males. Treatment 4, the negative control, consisted of aposymbiotic males and females. Each treatment group consisted of 30 mating pairs.

Every pair within each group was allowed to mate freely. Fecundity was recorded for each, measured as the number of eggs laid and proportion hatched. After one week, the bugs were all fed again, moved to new wells with the same mates, and fecundity was measured again. This was repeated for a third week. Three weeks after the pairs were placed together, individual bugs were whole-body extracted for DNA and underwent qPCR to determine final symbiont titers. Offspring resulting from each pairing were extracted for DNA and processed in like manner.

Results

Crosses with bed bugs maintained at elevated temperatures

Though there was some variation in exact measurements, all bugs from all the treatment groups tested positive for *Wolbachia*, including the negative control. This suggests that maintaining the bed bugs at 36°C for four weeks did not completely remove *Wolbachia*, and titers had rebounded to some extent during the four weeks after treatment. There was a significant difference in proportions testing positive for BLS when comparing normal females (0.85, n=20) and heat-treated females (0.20, n=20), or normal males (0.90, n=20) and heat-treated males (0.05, n=20) (Fishers Exact Test, $p<0.001$ for both). However, there was no statistically significant difference in the proportion of treated females testing positive for BLS when comparing between those mated with normal males (0.3, n=10) or treated males (0.1, n=10, p=0.582). The same applies to comparisons between treated males mated with normal or treated females (p=1).

The mean number of eggs laid and the proportion hatched per female within each treatment following the first and second feedings showed different results from week to week (Table 3.1). For the first feeding there was a significant effect of treatment on the mean number of eggs laid ($F(3,4)=37.47$, $p<0.01$) and on the proportion hatched ($F(3,4)=38.68$, $p<0.01$). The mean number of eggs laid per female had two groups within which the means were not significantly different, with females maintained at elevated temperature laying lower numbers of eggs than normal females (Figure 3.1). Normal females mated with normal males had significantly higher hatch rate compared to the

other groups, and treated females mated with normal males had a significantly lower hatch rate than the others (Figure 3.1).

After the second feeding there was a significant treatment effect on number of eggs being laid ($F(3,4)=10.40$, $p=0.02$), but the pattern of significant groups had changed and there was no longer a significant treatment effect on the proportions of eggs hatching ($F(3,4)=2.35$, $p=0.21$). There was a significantly higher number of eggs laid in Treatment 2 (normal females and treated males) compared to Treatment 3 (treated females and normal males) (Figure 3.2).

Crosses with bugs from symbiont free strains

All normal (NY1) males and females tested positive for *Wolbachia* after three weeks of mating, whether they were paired with aposymbiotic or normal mates. Likewise, all aposymbiotic line males and females tested negative for *Wolbachia* after three weeks of mating, regardless of symbiont status of the mate. Based on these results, there is no evidence to disprove the null hypothesis that the proportions being compared are equal (Fishers Exact Test, $p=1$). There is no indication that *Wolbachia* is transferred between males and females through traumatic insemination (Figure 3.3).

No males from aposymbiotic strains tested positive for BLS after mating with normal females (n=30). This was not significantly different from the proportion of aposymbiotic males which tested positive after mating with aposymbiotic females (0.04, n=28). As with *Wolbachia*, there is no evidence to disprove the null hypothesis that the proportions being compared are equal (Fishers Exact Test, $p=0.4828$). Likewise, there was no significant difference between the proportions of normal males testing positive for BLS after mating with normal females (0.77, n=30) or with aposymbiotic females (0.83,

p=0.748). There is no evidence that BLS is transferred to males from females (Figure 3.4).

Females from aposymbiotic lines all tested negative for BLS when mated with aposymbiotic males (n=28). However, when females from these same symbiont-free lines were mated with normal males, a significant proportion (0.57, n=30) tested positive for BLS (Fishers Exact Test, $p<0.01$), indicating that transmission of BLS from males to females had occurred (Figure 3.4).

The symbiont profile of the female was highly correlated with the symbiont profile of offspring for both *Wolbachia* and BLS ($p<0.01$). If the female was lacking one or both symbionts, her offspring also did not have it, regardless of male symbiont status. In addition, aposymbiotic females that apparently gained BLS from the males during mating did not transmit the symbiont to their offspring in the course of the experiment.

When the female was normal, the profile of the male had no significant impact on *Wolbachia* or BLS in the offspring. Offspring of normal females tested positive for *Wolbachia* regardless of its presence in the male mates ($p=1$), and the proportions of offspring that tested positive for BLS when the male was aposymbiotic (0.94, n=39) was not significantly different than the proportion testing positive when the male was also normal (0.89, n=26, Fishers Exact Test, $p=0.64$) (Figure 3.5).

While the symbiont status of males had no significant impact on the presence of symbionts in their offspring, there was a significant impact on the total offspring resulting from the each of the four treatments ($F(3,114)=57.14$, $p<0.01$). Further analysis of these results showed three significantly different groupings in the mean total offspring (Tukey HSD All-Pairwise Comparisons, $\alpha=0.05$). Mean offspring produced by normal females

with normal males (10.96, SD=2.91) was not significantly different than that produced by normal females with aposymbiotic males (10.12, SD=2.68), indicating that male symbiont status does not affect the overall fecundity of normal females. However, aposymbiotic females paired with normal males had significantly more offspring (6.41, SD=3.26) than those paired with aposymbiotic males (2.60, SD=1.76), but significantly less than normal females.

Discussion

Maintenance at elevated temperatures

The crosses with bugs exposed to 36°C for four weeks resulted in all sampled bugs from all treatments testing positive for *Wolbachia*. Since samples taken immediately after treatment were negative for both symbionts, it is likely that symbionts had been severely reduced to levels not detected by qPCR, and levels of *Wolbachia* subsequently rebounded while BLS did not. However, there was insufficient power in the experiment to determine if transmission of BLS took place. Similar complications using temperature to eliminate symbionts have been documented by Heaton (2013) in her dissertation work, in which she focused on impacts of *Wolbachia* in bed bugs and also used elevated temperatures in an attempt to produce aposymbiotic lines. In order to overcome only temporary decreases in *Wolbachia* titers, experiments using these bugs were limited to the first two weeks following treatment (Heaton 2013).

In my experiments, the fecundity data following the first feeding likely represents crosses with treated bugs having very low *Wolbachia* titers and lacking BLS. Treated females had lower fecundity than the normal females, but the results may be confounded with possible side-effects of exposure to higher temperatures which could lower

fecundity regardless of symbiont status since maintaining bed bugs at 36°C inhibits their development (Usinger 1966). At slightly lower temperatures, elevated temperature may be a viable option for ongoing elimination of symbionts. When spider mites were maintained at 32°C for six generations, they were cured of their *Wolbachia* endosymbiont (Van Opijnen and Breeuwer 1999). This type of long-term strategy has not been explored in bed bugs, as current strategies have focused on faster elimination. However, natural environmental changes in temperature were shown to impact the levels of *Wolbachia* infection in a tachinid fly (Guruprasad et al. 2011). If similar patterns were found in bed bugs and their symbionts, it might have an impact on the overall severity of bed bug infestations in a given geographic region, which do vary considerably (Potter et al. 2015).

The nature of interactions between an endosymbiont and host can be changed by altered symbiont titers. For example, in *Drosophila* a male-killing phenotype was no longer exhibited at low levels of *Wolbachia* whether the low levels were experimentally induced by elevated temperatures (Hurst et al. 2000) or the result of natural variation in symbiont titer (Unckless et al. 2009). Since *Wolbachia* in my experiment was not completely removed, the effects demonstrated may or may not be the same as complete elimination. Clear analysis of symbiont impacts on hosts is complicated by high natural variation in levels of symbionts in hosts, such as described in mosquitoes (Ahantarig et al. 2008) and *Drosophila* (Unckless et al. 2009).

The data from the second feeding after treatment with elevated temperatures represents treated bugs with normal levels of *Wolbachia* but lacking BLS. It is not completely clear what effect BLS might have on the number of eggs laid, as the two treatments pairing normal and aposymbiotic bugs were the only ones statistically

different from each other, but neither was different from the positive or negative controls. It may be that normal females lay more eggs when mated with BLS-free males than BLS-free females produce with normal males, but this could also be confounded by side-effects of treatment. The proportion hatching was not significantly different for any group, despite differences in BLS presence. It therefore appears that either the lower hatching proportions seen after the first feeding were due to treatment side-effects or low levels of *Wolbachia* rather than loss of BLS, or that BLS-free bugs have lower fecundity only initially.

Crosses with bugs from aposymbiotic strains

With respect to transmission of symbionts, the results are clear; *Wolbachia* was not transmitted horizontally through traumatic insemination, while BLS was transmitted to the female from the male at least some of the time (0.68 when aposymbiotic females were mated with males having BLS), but not from the female to the male. That proportion is nearly as high as the proportion testing positive with normal populations (0.75), and could be a major factor in maintaining BLS in the bed bug population if this infection were to be transmitted to offspring. However, it is also clear that BLS from the male was not transmitted in measurable amounts to offspring for at least the first three weeks. Perhaps this is because BLS had not yet migrated to the ovaries in the female after being introduced by the male. It is possible that given more time, BLS-positive offspring would have been produced by these females. Further studies should be done to verify whether transmission of BLS to offspring is exclusively maternal, as it seems to be with *Wolbachia*. There is also potential for studying movement of BLS through a population or patterns of mating in bed bugs by monitoring BLS infection over time.

In the short-term, aposymbiotic females experience higher fecundity if mating with normal males. Various explanations for this result are possible. Since it has been demonstrated that ejaculate components improve female fecundity (Reinhardt et al. 2009) and seminal fluid availability can vary (Reinhardt et al. 2011), better health and vigor of normal males could be the answer. A variation on this theme is the possibility that some nutrients being supplied through the ejaculate are provided by symbionts in the male. Alternatively, transmission of BLS to the female could restore direct benefits of the symbiosis. These hypotheses have not been tested.

The long-term impacts of the symbionts on fecundity in bed bugs are cloudier, given the changes shown in the different groups over time. Initially, groups in which the female did not have symbionts had much lower fecundity, but both number of eggs laid and proportion hatch increased the following two weeks. While I only gathered data for the first three weeks, it is possible that the number of eggs laid and proportion hatched would eventually have been about the same for all treatment groups. By the third week the number of eggs being laid and proportion hatched in Treatment 3 (aposymbiotic females and normal males) was not significantly different than the normal female groups. While it is easy to account this improvement in fecundity to the associated transmission of BLS, results can be misleading. Treatment Group 4 also showed improved fecundity despite total absence of symbionts, and life table analysis of bed bugs shows that mean female fecundity increases over the first five weeks of reproduction (Polanco et al. 2011).

BLS seems to be readily transmitted from males to females during mating. No BLS was detected in the resulting offspring, indicating that it was not transmitted to offspring by the males during the first three weeks after pairing. Over time the newly

infected females might begin to transmit BLS to their offspring. Venereal transmission of many pathogenic viruses, fungi, protists and bacteria have been demonstrated in a variety of arthropod organisms (Knell and Webberley 2004, Mavale et al. 2010), and another study reports Rickettsial symbionts being transmitted from males to subsequent offspring in this manner (albeit through typical and not traumatic insemination) (Brumin et al. 2012).

Bed bugs have the potential to vector Chagas Disease (Salazar et al. 2015), but one reason it is less likely to occur outside of the lab is the lack of pathogen transmission from parent to offspring. Traumatic insemination as a route of horizontal transmission should be evaluated for other diseases which could be carried and vectored by bed bugs, but is only likely if the pathogens are able to invade the reproductive organs of the male.

Table 3.1. Results of reciprocal crosses between normal bed bugs and those maintained at 36°C for four weeks, showing subsequent fecundity. The second feeding took place two weeks after the first. Numbers shown are mean ± SEM. N=2 for all treatments. **Treatment 1:** normal females with treated males (positive control). **Treatment 2:** normal females with treated males. **Treatment 3:** treated females with normal males. **Treatment 4:** treated females and males (negative control).

Treatment	Feeding	Eggs laid	Proportion Hatched
F+M+	1	15.72 ± 0.05	0.94 ± 0.02
	2	8.21 ± 0.44	0.90 ± 0.04
F+M-	1	17.46 ± 0.23	0.55 ± 0.09
	2	9.98 ± 0.06	0.96 ± 0.02
F-M+	1	10.33 ± 1.03	0.24 ± 0.04
	2	6.56 ± 0.59	0.88 ± 0.02
F-M-	1	12.30 ± 0.10	0.71 ± 0.02
	2	8.46 ± 0.46	0.89 ± 0.03

Figure 3.1. Fecundity after first feeding for reciprocal crosses of normal bed bugs and those maintained at 36°C for four weeks. **Treatment 1:** normal females with normal males (positive control). **Treatment 2:** normal females with treated males. **Treatment 3:** treated females with normal males. **Treatment 4:** treated females and males (negative control). Bars show standard error of the mean. Letters over each bar indicate groups with statistically significant differences between means, Tukey HSD All-Pairwise Comparisons α=0.05, n=2 for each treatment group.

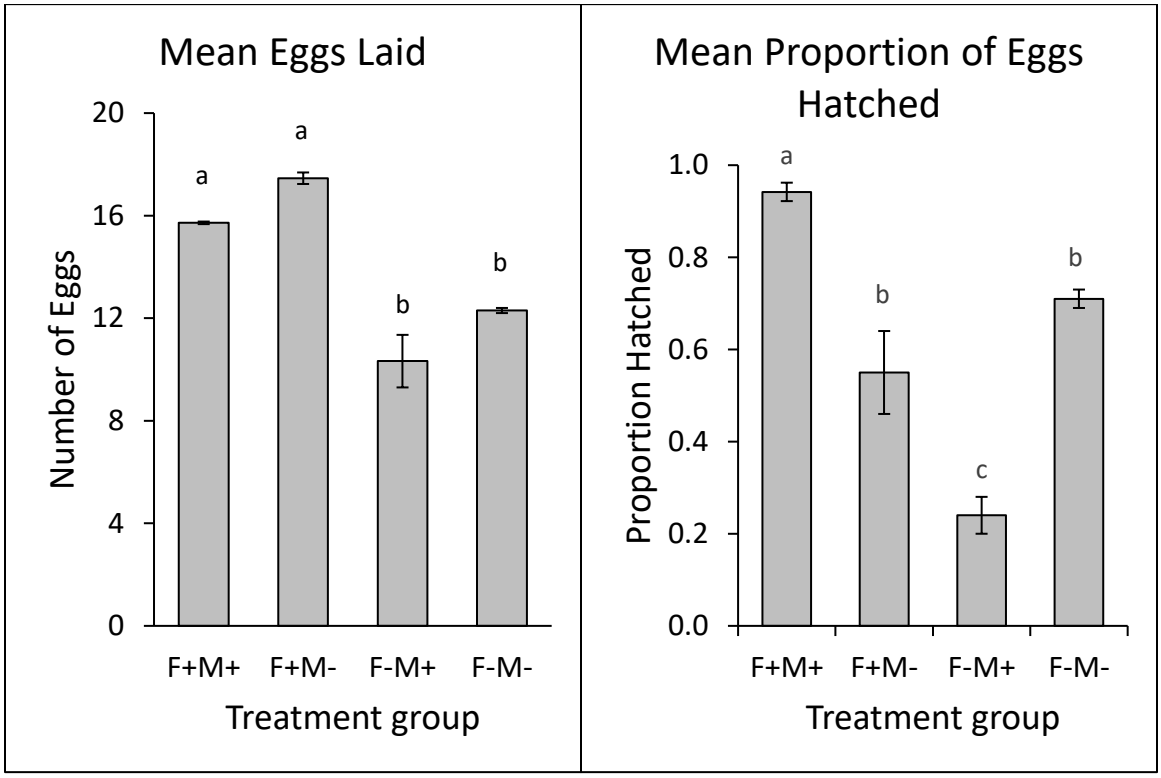


Figure 3.2. Fecundity after Second feeding for reciprocal crosses of normal bed bugs and those maintained at 36°C for four weeks. **Treatment 1:** normal females with treated males (positive control). **Treatment 2:** normal females with treated males. **Treatment 3:** treated females with normal males. **Treatment 4:** treated females and males (negative control). Bars show standard error of the mean. Letters over bars indicate groups with statistically significant differences between means, Tukey HSD All-Pairwise Comparisons α=0.05, n=2 for each treatment group.

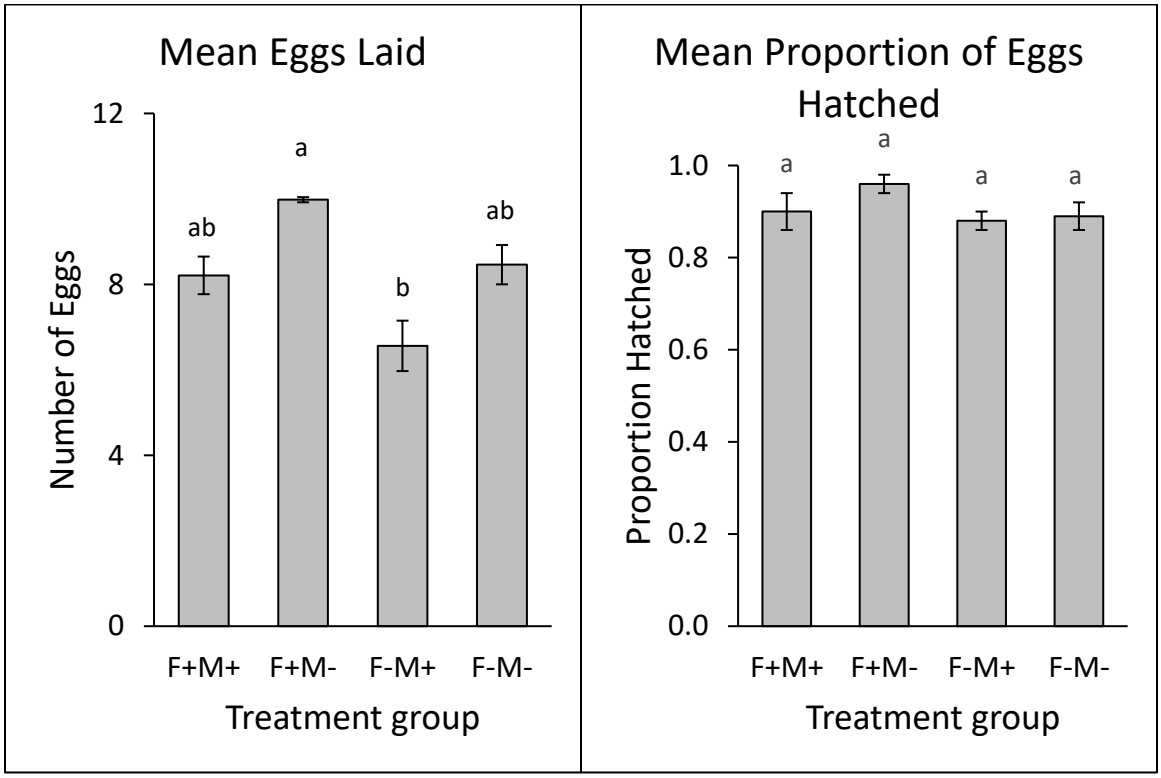


Figure 3.3. Proportion of individual bugs testing positive for *Wolbachia* following reciprocal crosses of normal bed bugs with aposymbiotic strains. **Treatment 1:** normal females with normal males (positive control). **Treatment 2:** normal females with aposymbiotic males. **Treatment 3:** aposymbiotic females with normal males. **Treatment 4:** aposymbiotic females and males (negative control). All females tested positive for *Wolbachia* in treatment 1, while no females tested positive for *Wolbachia* in treatments 3 and 4. All males tested positive for *Wolbachia* in treatment 1, and no males tested positive in treatments 2 and 4. Bars show standard error of the mean, n=30 males and 30 females for treatments 1, 2 and 3, and n=28 males and 28 females for treatment 4.

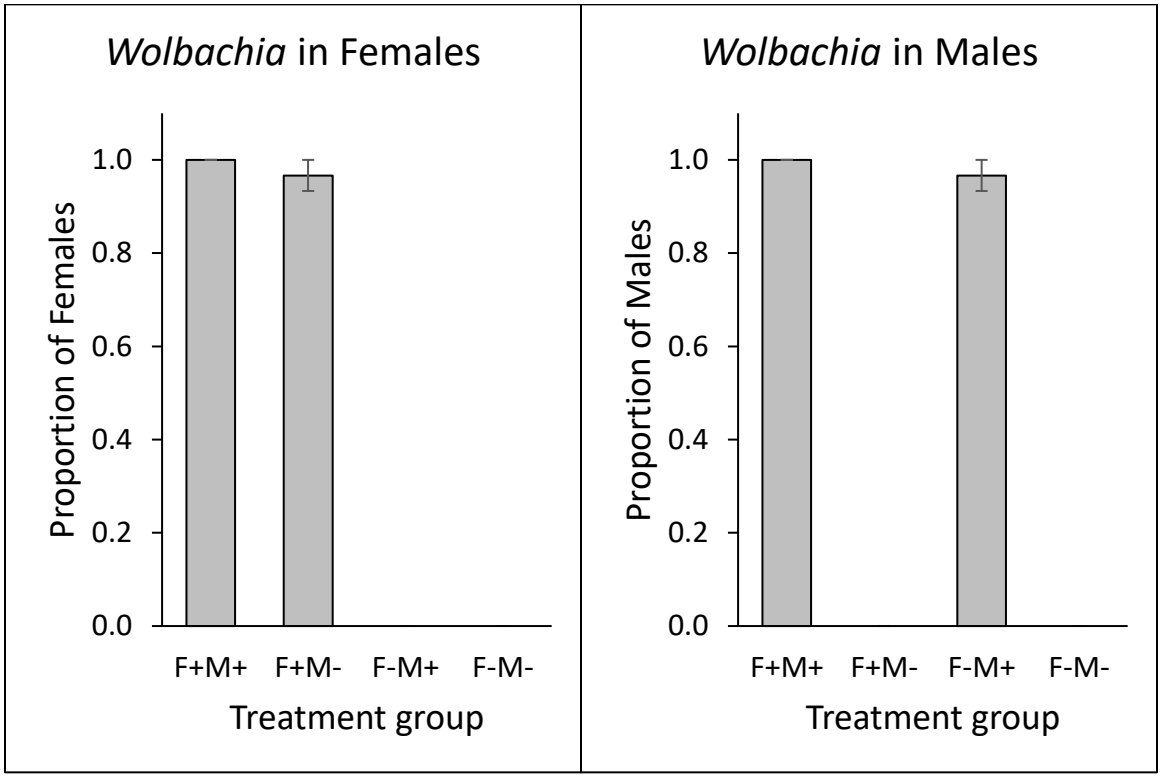


Figure 3.4. Proportion of individual bugs testing positive for BLS following reciprocal crosses of normal bed bugs with aposymbiotic strains. **Treatment 1:** normal females with normal males (positive control). **Treatment 2:** normal females with aposymbiotic males. **Treatment 3:** aposymbiotic females with normal males. **Treatment 4:** aposymbiotic females and males (negative control). No females tested positive for BLS in treatment 4, and no males tested positive in treatment 2. Bars show standard error of the mean, n=30 males and 30 females for treatments 1, 2 and 3, and n=28 males and 28 females for treatment 4.

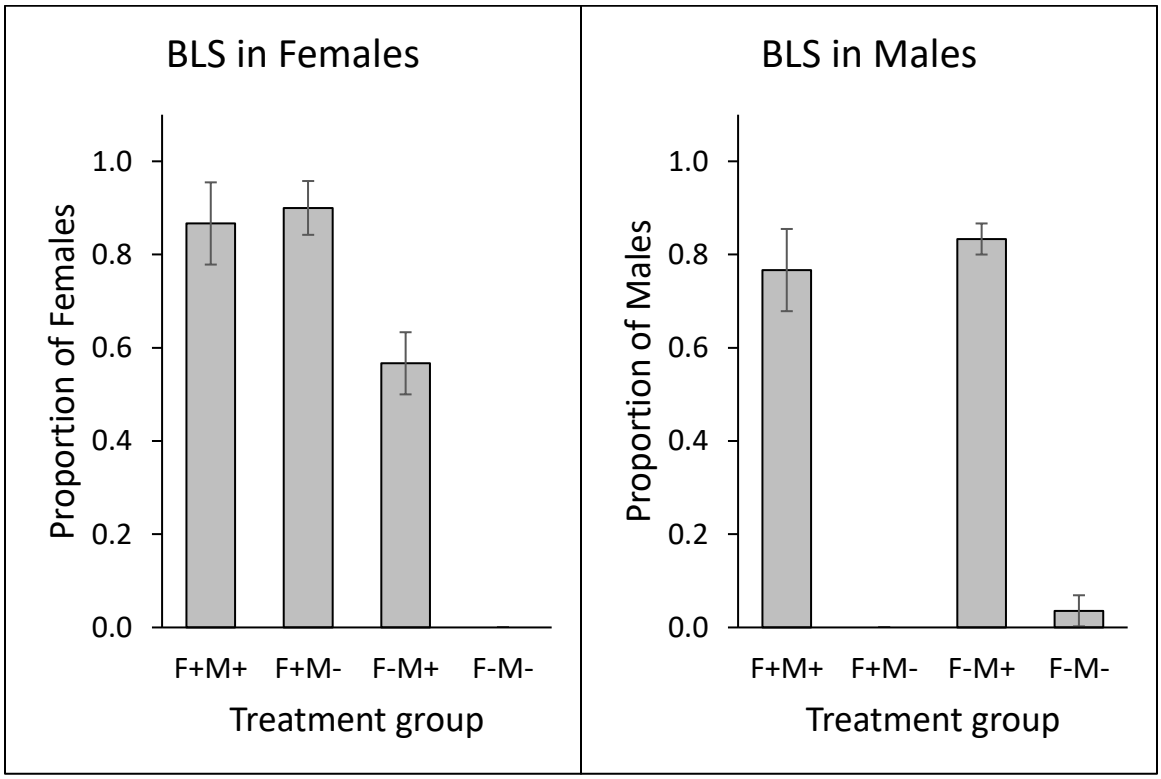


Figure 3.5. Proportions of offspring from reciprocal crosses testing positive for endosymbionts. **Treatment 1:** normal females with normal males (positive control), n=24. **Treatment 2:** normal females with aposymbiotic males, n=26. **Treatment 3:** aposymbiotic females with normal males, n=25. **Treatment 4:** aposymbiotic females and males (negative control), n=25. All offspring in treatment 1 were positive for *Wolbachia*. No offspring in treatment groups 3 or 4 tested positive for either symbiont. Bars show standard error of the mean.

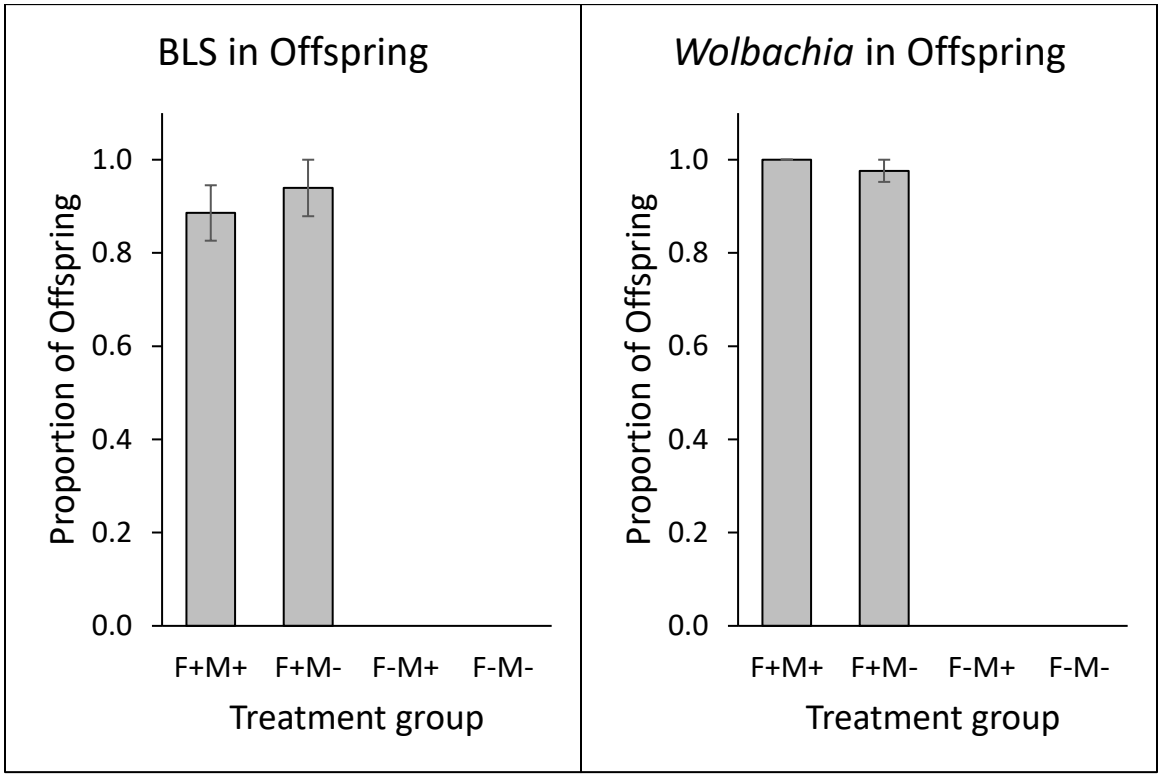
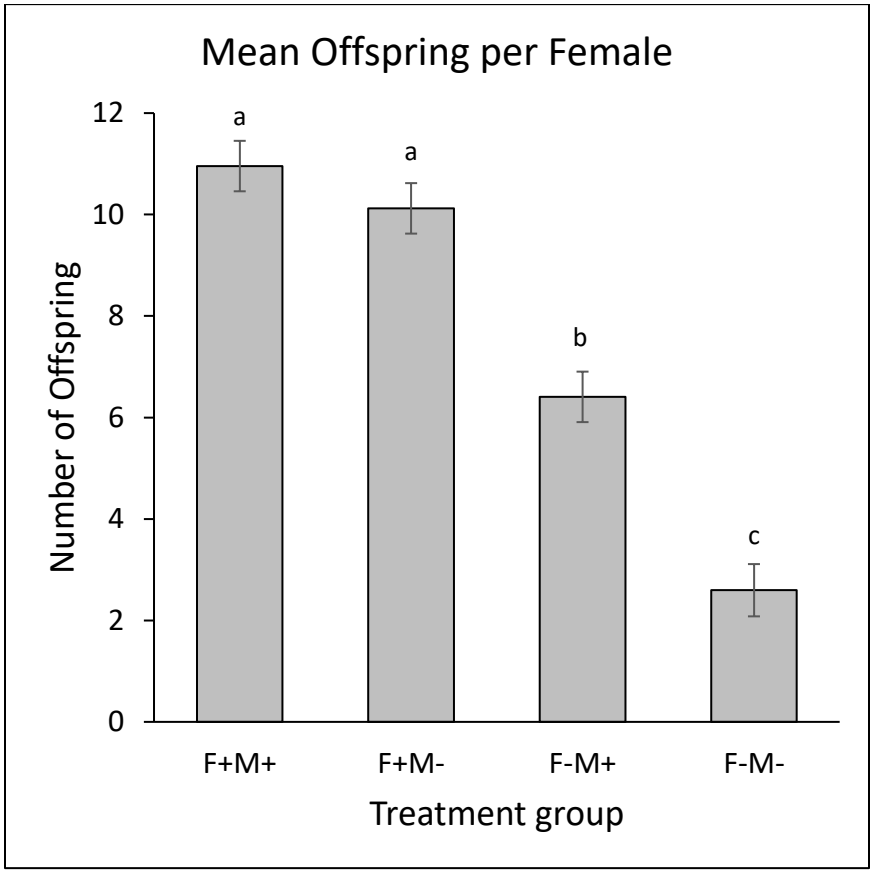


Figure 3.6. Mean number of offspring produced following reciprocal crosses between normal and aposymbiotic bed bugs. **Treatment 1:** normal females with normal males (positive control), n=30. **Treatment 2:** normal females with aposymbiotic males, n=30. **Treatment 3:** aposymbiotic females with normal males, n=30. **Treatment 4:** aposymbiotic females and males (negative control), n=28. Bars show standard error of the mean. Letters indicate groupings of statistically significant difference, Tukey HSD All-Pairwise Comparisons, $\alpha=0.05$.



Chapter 4: The impacts of endosymbiont elimination on development in the bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae)

Introduction

Endosymbiosis is a special case of symbiosis in which one organism lives inside another dissimilar organism (the host). These relationships occur on a continuum, ranging from mutualistic interactions in which both host and symbiont benefit from the relationship to parasitism in which the symbiont benefits to the detriment of the host (Moran et al. 2008). They may also be described in terms of organismal interdependence: obligate endosymbiosis, in which the host and/or endosymbiont depend on the relationship for survival, or facultative, in which the relationship is not necessary for at least one of the pair (Moran et al. 2008). Multiple endosymbiotic organisms may exist within a single host, resulting in more complex interactions (Wu et al. 2006, Moran et al. 2008).

The interactions of microbial endosymbionts with their hosts have received increasing attention as improved molecular technology provides new ways to measure and detect them. Some of the interactions more recently described or discovered include morphological changes such as color and size (Tsuchida et al. 2010), reproductive manipulation such as egg production (Dedeine et al. 2001), reduced sperm competitive ability (de Crespigny and Wedell 2006), increased mating behaviors (de Crespigny et al. 2006), cytoplasmic incompatibility and male-killing (Bourtzis and O'Neill 1998), provisioning of B vitamins and amino acids to insects on nutrient-limited diets (Nakabachi and Ishikawa 1999, Brownlie et al. 2009), defense against natural enemies (Oliver et al. 2003, Brownlie and Johnson 2009), insecticide resistance (Kikuchi et al.

2012), immune function and protection (Scarborough et al. 2005, Haine 2007), influences on food choices (Leonardo et al. 2003, Tsuchida et al. 2004), vector competency (Beard et al. 1998, Beard et al. 2002) and so on. It is clear that many symbionts have shown and continue to demonstrate an impact on the biology of arthropods, and bed bugs are no exception.

In 1997, Hypsa and Aksoy were the first to sequence bacterial genes extracted from bed bugs. They identified the two symbionts present as a *Wolbachia* sp. in the F clade and a gamma-proteobacterium referred to as BEV-Like Symbiont (BLS) based on similarities to a symbiont found in sharpshooters (Campbell and Purcell 1993). These identities have been confirmed by multiple researchers (Rasgon and Scott 2004, Sakamoto and Rasgon 2006a, Hosokawa et al. 2010, Meriweather et al. 2013), along with evidence that these two symbionts were found consistently in bed bug populations around the world (Sakamoto and Rasgon 2006b, Sakamoto et al. 2006, Siddiqui and Raja 2015, Akhoundi et al. 2016).

It has long been accepted that in order to thrive, arthropods that feed exclusively on blood require symbionts to provide supplemental nutrients including vitamins and amino acids (Koch 1960, Brooks 1964, Chang 1975). This has been demonstrated in aphids, tsetse flies, lice, and others (Nakabachi and Ishikawa 1999, Brownlie et al. 2009, Rio et al. 2016). This host-symbiont interaction remained unproven in bed bugs until Hosokawa et al. (2010) published a study that demonstrated that *Wolbachia* played a major role in providing nutrients to the bed bug, without which they experienced decreased fecundity and slowed development. This was a significant finding, since most *Wolbachia* infections previously studied in other arthropod hosts had demonstrated

reproductive manipulation, not benefits (Werren 1997, Werren et al. 2007, Rio et al. 2016). The discovery of nutritional provisioning by *Wolbachia* is not surprising, since the C, D and F clades of *Wolbachia* provide similar benefits in nematode and other arthropod hosts (Bandi et al. 2001, Nikoh et al. 2014) as well. In subsequent studies, it has been confirmed that loss of *Wolbachia* in bed bugs caused negative effects on the host such as lower fecundity in both male and female bugs (Heaton 2013). Sequencing the genome of *Wolbachia* in bed bugs also revealed that this symbiont carries the genes necessary for producing the vitamins Riboflavin and Biotin (Nikoh et al. 2014).

The present study approaches a similar question about symbionts in bed bugs, but with some key differences. First, while the above studies investigated the role of *Wolbachia*, there has been little study done on BLS, the secondary symbiont. Multiple endosymbionts can interact in a single host (Wu et al. 2006), and my experiments include both symbionts.

Second, previous studies used bed bugs that were currently being treated with antibiotics or elevated temperatures or were the offspring of treated bugs. The present study attempts to avoid confounding treatment effects by using bugs two generations removed from treatments for eliminating symbionts. While many potential impacts need to be explored, I have focused in this study on the impacts of symbiont loss on host fecundity, development time, and size of adults.

Materials and methods

Bed bugs used in these experiments came from a colony (NY1) that originated in Plainview, NY, in April 2007. This colony has been shown to exhibit insecticide resistance to pyrethroids (Zhu et al. 2010; Gordon et al. 2014). It was maintained in the

laboratory under standard conditions of 26.7° C, 65 ±5% RH, and a photoperiod of 14: 10 (L: D) h. The colony was fed weekly on a parafilm-membrane feeding system with defibrinated rabbit blood (Quad Five, Ryegate, MT) heated to 34° C by a circulating water bath (Montes et al. 2002). Virgin adult bugs were obtained by isolating recently-fed fifth instar nymphs. Bugs for all experiments were used 7–14 days after molting to the adult stage.

DNA extractions for all experiments were completed with DNAzol (Thermo Fisher Scientific, Inc., Waltham, MA) using a modified version of the standard protocol. The protocol for successful extraction required close adherence to specific requirements. First, place the sample to be extracted into a microcentrifuge tube with 100µl of DNAzol. Homogenize, then let stand for 15 min. Centrifuge at 10,000 g for 10 min, then remove supernatant to a new tube with 45µl of 100% ethanol and mix thoroughly. Let stand for 3 min, then centrifuge at 16,000 g for 15 min. As a wash to remove contaminants, discard supernatant and pipette 160 µl of 75% ethanol into the tube. Mix well, then centrifuge for 5 min at 16,000 g. Repeat with a second wash and centrifuge, then remove and discard supernatant. Finally, place 50 microliters of PCR-quality water into each tube and mix well. Keep extracted DNA frozen until use.

All extractions were verified and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham MA) to measure DNA concentration and monitor for quality of product. Template was prepared for qPCR by dilution in pure water to produce template samples with equivalent DNA concentrations of 5 ng/µl.

I used an iCycler iQ Real Time PCR Detection System (BioRad Laboratories, Inc., Hercules CA) with 10 µl reactions (4.7 µl template, 5 µl FastStart SYBR Green Master Mix (Roche Life Science, Penzberg Germany), 0.3 µl primers). A melting curve was included as an indicator to verify amplification of a single pure amplicon. All qPCR followed a standard protocol (denature for 30 s at 95°C, anneal primers for 15 s at 55°C, DNA extension for 30 s at 72°C, plate read, 40 cycles) followed by a melting curve from 55°C to 95° C.

Determining levels of both BLS and *Wolbachia* 16S genes with qPCR required three reactions for each sample using appropriate primers. Primers for BLS and *Wolbachia* were designed from existing 16S sequence data on GenBank (accession numbers U65654 and AY316361.1, respectively) using the NCBI primer-designing service. The successful primers were as follows: for *Wolbachia* (859F 5'GACCCGCACAAGCGGTGGAG 3', 973R 5'AATCCGGCCGAACCGACCCT 3'), for BLS (74F 5'AGCGGCGGACGGGT-GAGTAA 3', 194R 5'GCGTGAAGCCCGAAGGTCCC 3') and for the reference gene Clrpl8 (accession no. EZ419784, Clrpl8F 5'AAAGGCACGGTTACATCAAAGGTG 3', Clrpl8R 5'TAGTCTTGAACCTATAGGGGTCCC 3').

All from qPCR was processed using the LinRegPCR software program (Ruijter et al. 2009). This program performs baseline corrections on the raw fluorescence data gathered during the reactions, then uses linear regression analysis to determine PCR efficiency for each. Calculation of initial concentrations of amplicons in each sample, measured in arbitrary fluorescence units (N_0 values), is made using quantitation cycle (Cq) values and mean PCR efficiency per amplicon. The N_0 values resulting from this

processing with LinRegPCR were calibrated using the reference gene Clrpl8 to control for variation in the initial total template concentration. The resulting values were $\log_2$ transformed for normality in statistical analysis.

Results were analyzed with Statistix 9.0 (Analytical Software, Tallahassee FL).

Symbiont effect on adult size

Aposymbiotic strains were created by taking virgin adult bugs from NY1 colony and feeding them weekly for eight weeks on blood containing rifamycin. Following treatment, they were allowed to mate and the resulting aposymbiotic offspring (confirmed by random sampling and qPCr of nymphs) were used as the basis for a new strain. Control strains were created simultaneously by taking bugs from NY1 and feeding them for eight weeks on normal blood.

Second generation (F2) adult bed bugs from three aposymbiotic strains and control strains were used to test if loss of symbionts had an effect on bed bug body size. For 32 male and 32 female bugs from each replicate, I measured the head capsule at its greatest width at the eyes and the total body length from the head to the tip of the abdomen (unfed) using a dissecting microscope. Adults were identified by full sclerotization of the cuticle and the presence of wing pads. Males and females were differentiated by the presence of external male genitalia (left paramere) with the abdomen coming to a relative point, while females were more symmetrically rounded in shape, lacked the paramere, and had an ectospermalege. After measuring, each individual was whole-body extracted for DNA and the symbiont titers were determined using qPCR. The measurements of aposymbiotic bugs were compared to those of control bugs with normal symbiont profiles.

Symbiont effects on development time

In order to determine the effect of symbiont loss on the amount of time required for successive generations to develop, I recorded the length of time required for bed bug individuals to develop from egg to adult. Three aposymbiotic lines and three control lines were measured, with weekly feedings. Notes on development were taken after each feeding, including whether or not molting occurred, mortality, and proportion of individuals which fed.

Symbiont effects on nutrition

Hosokawa et al. (2010) indicated that *Wolbachia* was a nutritional obligate, and found that by supplementing the diet of aposymbiotic bugs with B vitamins they could restore them to normal fecundity. With preliminary evidence suggesting that aposymbiotic bugs take longer to develop, I investigated whether vitamin supplements would restore normal development time. To test this hypothesis, I added the vitamins thiamine (100 µg/ml), riboflavin (20 µg/ml), niacin (100 µg/ml), folic acid (30 µg/ml), pantothenic acid (100 µg/ml), and pyridoxine (100 µg/ml) to blood meals, following the protocol set forth by Lake and Friend (1968). Using first, second and third instar nymphs from aposymbiotic and control strains, I measured the time required for development to the adult stage. Sampled nymphs were split into two groups, with one half feeding on supplemented and the other normal blood. After feeding, they were placed in individual wells on a 24-well cell culture plate for observation. The time required for molting after feeding was then compared for those fed on vitamin-supplemented and normal blood.

Results

Symbiont effect on adult size

There was a significant effect of symbiont presence on both head capsule size and total body length, as both female and male bed bugs without symbionts were significantly smaller on average than their normal counterparts (female head width $F(1,72)=69.20$, $p<0.01$; female body length $F(1,72)=54.91$, $p<0.01$; male head width $F(1,76)=10.06$, $p<0.01$; male body length $F(1,76)=6.22$, $p=0.02$; Table 4.1, Figures 4.1, 4.2). The size differences were greater for female bed bugs, which were on average 8.45% smaller when measured at the head capsule and 9.61% shorter in total length when they developed without symbionts. Male aposymbiotic bugs were about 3.49% smaller than controls with regards to head capsule 3.96% shorter in total length.

While the mean sizes of the groups were significantly different, overlap in size distribution between groups was notable, with some aposymbiotic adults being larger than some normal adults (Figures 4.1, 4.2).

Symbiont effects on development time

Symbiont loss significantly slowed development time. Control development from egg to adult required seven weeks, and three generations were completed in 24 weeks. However, there was slower development for the aposymbiotic lines. The first-generation offspring following treatment (F1) required 10 weeks to develop to adults in all three replicates, and the second generation (F2) required 26 ± 10.8 wk to become adults (Table 4.2). No third generation aposymbiotic bugs reached adulthood during the course of these experiments despite a full year of weekly feedings.

Because development was measured in weeks and feeding was on a weekly schedule, there was no measured variation in the length of time required for F1 aposymbiotic strains or the controls to reach adulthood. While this and the small number of replicates limit statistical analysis, Wilcoxon Rank-Sum analysis indicates that aposymbiotic bugs took a significantly longer time to develop (α=0.05) for both the F1 and F2 generations.

Symbiont effects on nutrition

Times required to molt after feeding are given in Table 3. There was a significant effect of instar, supplement treatment, and aposymbiotic status on the time required to molt. There were also significant interactions between factors, with supplement treatment by instar and by aposymbiotic status both significant (Three-way Analysis of Variance, Table 4.3). The mean time required to molt was not significantly different between control bugs fed normal or supplemented blood, but aposymbiotic bugs given vitamin supplements molted sooner on average than aposymbiotic bugs given normal blood (Figure 4.3). However, aposymbiotic bugs still took significantly longer to develop than normal bugs even when given supplements.

Discussion

Endosymbionts have been shown to have many impacts on their host. When the diet of the host is restricted to a nutritionally deficient source, such as xylem or blood, it is predicted that at least one symbiont will fulfill a nutrient-provisioning role (Zeintz et al. 2004, Moran 2008). Studies have shown that this is the case with *Wolbachia* in bed bugs, which showed delayed development when the symbiont was removed (Hosokawa et al. 2010, Heaton 2013, Nikoh et al. 2014). This study confirms these results, as I also

found delayed development when the symbionts were eliminated. Vitamin supplements succeeded in reducing that delay, but did not restore normal development. The reason for this difference may be that biotin was not included as a supplement in my experiments. In a study published after the conclusion of this work, Nikoh et al. (2014) showed that *Wolbachia* in bed bugs has the genetic pathway for biotin production and that it is the key nutrient provided to the bed bug by this symbiont.

It is likely that the reduced size of aposymbiotic adults detailed here is a result of nutritional deficiency, similar to that seen in other insects (Nakabachi and Ishikawa 1999, Brownlie et al. 2009). However, the cause of reduced fecundity is among the same bugs is not so clear cut. The slightly smaller size of aposymbiotic males probably has little effect on their physical ability to mate with females, since all males in my studies were successful in mating with females regardless of size, and mating behaviors did not appear challenged. Male mating rate has been correlated with amount of seminal fluid available (Reinhardt et al. 2011), but this study did not provide male bed bugs with multiple females nor measure sperm.

However, the nearly 10% smaller size of aposymbiotic females could directly impact the overall fecundity of aposymbiotic bed bug strains. Body size in female insects demonstrates a strong positive relationship, with each percent increase in body weight correlating with approximately one percent increase in fecundity (Honěk 1993). While it remains to be tested, smaller body size could result in smaller blood meals, reduced nutrient availability, fewer ovarioles, or lower storage capacity as eggs develop within the abdomen. It is also possible that symbionts affect reproduction independent of body size or nutrition, but this seems unlikely given the results of other researchers who showed a

return to normal fecundity with vitamin supplements for female bed bugs (Hosokawa et al. 2010, Nikoh et al. 2014).

While potential reduced fecundity may impact aposymbiotic strains, it probably does not constitute the limiting factor in population growth. Almost certainly, the effects of symbiont loss on development time far outweigh adult size in this regard. The effect of symbiont loss on the time required for bugs to become adults was significant, with aposymbiotic bugs requiring longer than normal bugs to develop completely. Interestingly, the development time for symbiont-free bugs increased with each generation, until individuals of the third generation did not develop to the adult stage at all during our experiments. Instead, they slowly progressed through the first three molts before apparently stalling in their development. Eventually some did reach the imago, but not until experiments had concluded, and data is not available for them. With development times lasting over six months, this effect is biologically significant in terms of population viability since this is well over 143 days, the mean life expectancy of bed bugs (Polanco et al. 2011).

As noted above, the relationships and interactions between endosymbiont and hosts are complex and diverse. I have confirmed that the loss of both *Wolbachia* and BLS in bed bugs results in reduced body size and increased development time, reduced to some extent by dietary supplements of B vitamins. While other work has focused on the role of the primary symbiont, *Wolbachia*, little is known about BLS. Koga et al. (2003) showed that when the primary and nutritional symbiont *Buchnera* is removed from aphids, a facultative symbiont can fulfil the nutritive role in its place. Other potential impacts on the host remain to be tested, particularly for BLS. These possible impacts

include reduced sperm competitive ability (de Crespigny and Wedell 2006), increased mating behaviors (de Crespigny et al. 2006), insecticide resistance (Kikuchi et al. 2012), and immune function and protection from pathogens (Scarborough et al. 2005, Haine 2007).

Table 4.1 The effect of symbionts on adult size. Head capsules were measured at the point of greatest width between the eyes, and body length was measured from the front of the head to the tip of the abdomen. Statistical analysis using Tukey All-Pairwise mean comparisons, α=0.05. Mean ±SEM.

	Normal	Aposymbiotic	%Δ	P-value
Head width (mm)				
Male	0.63±0.004	0.61±0.004	-3.49%	p<0.01
Female	0.68±0.003	0.62±0.007	-8.45%	p<0.01
Body Length (mm)				
Male	4.17±0.03	4.00±0.04	-3.96%	p=0.02
Female	4.48±0.03	4.05±0.06	-9.61%	p<0.01

Table 4.2 The effect of symbiont loss on development time from egg to adult over three generations. *Measurements of development were discontinued after 30 weeks in the F2 generation.

		Development Time for generations (wk)		
Treatment	Replicate	F1	F2	F3
Rifamycin	1	10	23	NA
	2	10	20	NA
	3	10	30*	NA
Control	1	7	7	7
	2	7	7	7
	3	7	7	7

Table 4.3 Analysis of Variance Table for effects of supplemental diet (Sup), Instar, and symbiont presence (Apo) on development time in bed bugs.

Source	DF	SS	MS	F	P
Instar	2	23.26	11.63	8.92	0.0001
Sup	1	29.95	29.953	22.98	0
Apo	1	823.56	823.564	631.94	0
Sup*Apo	1	37.35	37.352	28.66	0
Instar*Sup*Apo	6	32.76	5.461	4.19	0.0003
Error	2407	3136.88	1.303		
Total	2418				

Figure 4.1 Box and whisker plots showing the impact of symbiont loss on adult total body length.

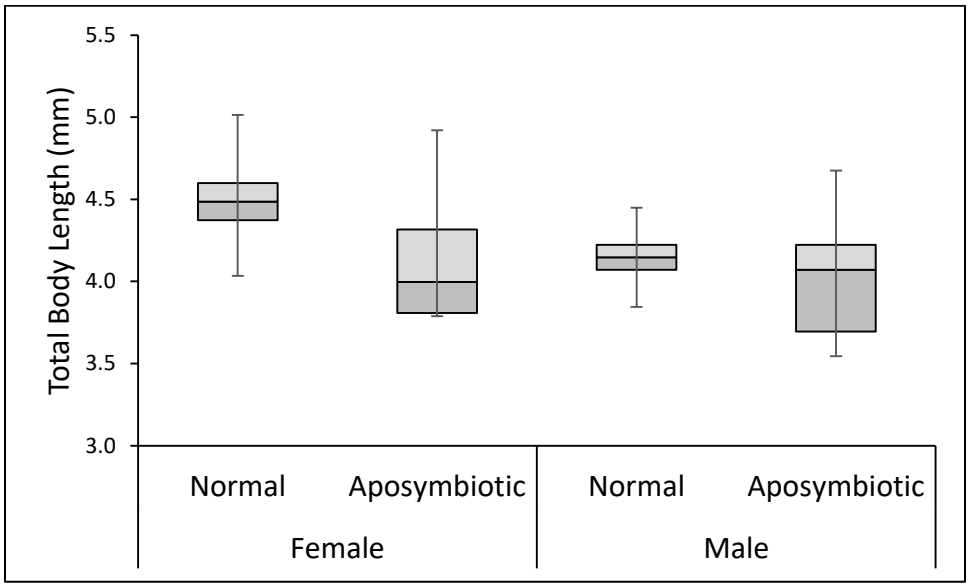


Figure 4.2 Box and whisker plots showing the impact of symbiont loss on adult head capsule width.

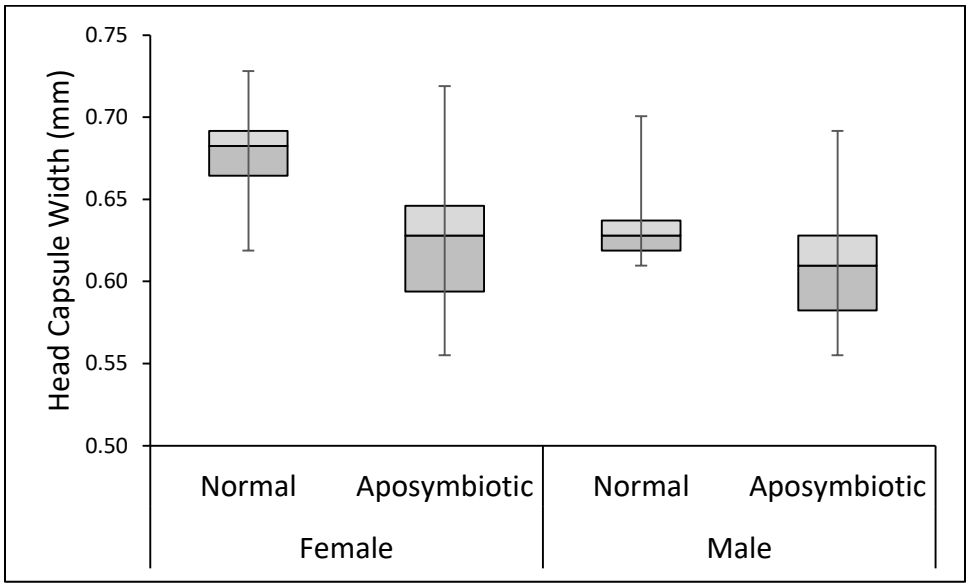
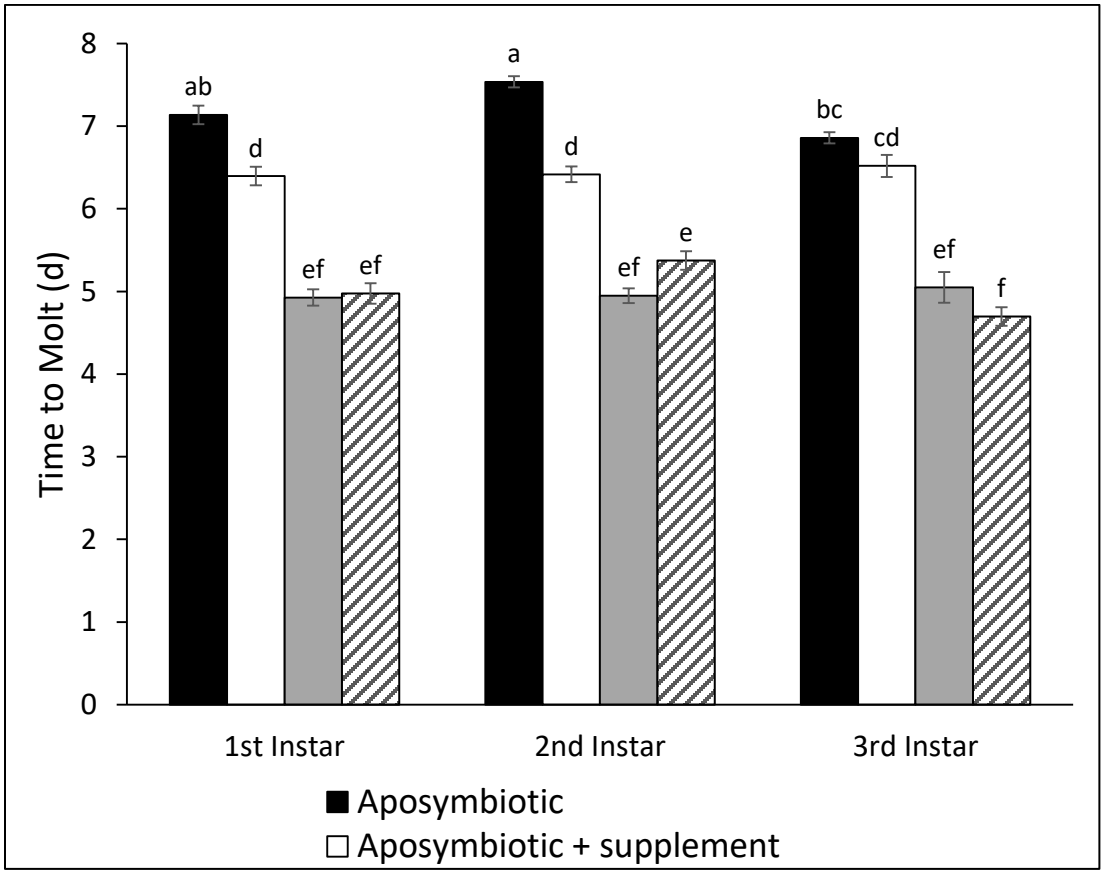


Figure 4.3 The effects of vitamin supplements on time required to molt after feeding. Sample size of treatments from left to right: first instar 102, 99, 305, 224, second instar 272, 220, 336, 271, third instar 290, 257, 24, 19. Bars represent SEM, letters indicate groupings of statistically significant difference (Tukey HSD all-Pairwise Comparisons, α=0.05).



Chapter 5: Effects of Juvenile Hormone Analog Formulations on Development and Reproduction in the Bed Bug *Cimex lectularius* L. (Hemiptera: Cimicidae)

Introduction

The following summarizes previously published research (Goodman et al. 2013). Bed bugs (*Cimex lectularius* L.) are one of the most commonly reported pest problems in many urban areas, and are often difficult to control (Potter et al. 2015). The cryptic nature of the bugs, the intimate settings where infestations occur, and widespread resistance to pyrethroids (Romero et al. 2007, Zhu et al. 2010) make eradication difficult even for professional pest control operators. Bed bugs are likely to take refuge on and around beds, couches, and other places where humans make frequent and prolonged contact, and consequently, where relatively few effective insecticides are labeled for use. Those that are most effective for bed bug treatment are valued for their residual action and low mammalian toxicity.

Juvenile hormone analogs (JHA) such as methoprene and hydroprene are attractive management options because of their low toxicity to mammals and their history of effectively controlling a variety of urban insect pests, such as cockroaches, mosquitoes, fleas, and stored product pests (Mohandass et al. 2006, Henrick 2007). Juvenile hormone (JH) plays an integral role in insect molting, development, and reproduction. By imitating abnormally high levels of JH, these analogs interfere with the development of normal reproductive adults. Gentrol® (Central Life Sciences, Schaumburg IL), with (*S*)-hydroprene as the active ingredient, is one JHA product labeled for use in bed bug management and is utilized extensively (Potter et al. 2015).

However, despite high usage, the efficacy of JHA formulations for bed bug control remains unclear.

Previous work with (*S*)-hydroprene on a laboratory colony found no impact on development time, but did show adult mortality (Todd 2006). In another study bed bug nymphs and adults were exposed to dry residues of (*S*)-methoprene, and demonstrated high efficacy against both laboratory-maintained bugs and insecticide-resistant field strains (Naylor and Boase 2008). However, the application rates used in the study were high, up to 15 times higher than the label rate of current methoprene-containing insecticides. Earlier work also showed methoprene efficacy against treated nymphs (Shaarawi et al. 1981, 1982) and eggs (Takahashi and Ohtaki 1975) only at similarly high rates.

In order to determine efficacy of Hydroprene and Methoprene for bed bug control, bed bugs of different stages originating from pyrethroid-resistant and susceptible colonies were exposed to aqueous dilutions of Gentrol® IGR Concentrate, and Precor® IGR Concentrate (Central Life Sciences, Schaumburg IL, active ingredient: (*S*)-methoprene) both as a direct spray of insects and dry residue in which bed bugs were confined to the treated surface.

Materials and Methods

Insects and materials

Three strains of bed bugs were tested. The FD colony came from a collection made in Ft. Dix, NJ, in 1974 (Bartley and Harlan 1974), and having long been maintained in the laboratory, is highly susceptible to various insecticides, including pyrethroids (Romero et

al. 2007). CIN-1 was initiated from over 200 bugs collected in an apartment in Cincinnati, OH in 2005. Relative to the long-maintained laboratory strain (FD), this colony is highly resistant to two pyrethroids (deltamethrin and λ-cyhalothrin) as measured by a residual assay with technical grade insecticide (Romero et al. 2007). NY-1 was collected from an infested apartment in Plainview, NY in April 2007, and has shown resistance to a pyrethroid (deltamethrin) measured by a residual assay and compared to the FD strain (Zhu et al. 2010).

Insects were housed in incubators at 26.7° C, 65 ± 5% RH, and a photoperiod of 14: 10 (L: D) h. All colonies were maintained on citrated rabbit blood using the methods of Montes et al. (2002) with a weekly feeding regimen.

The efficacy of (*S*)-methoprene and (*S*)-hydroprene formulations were tested using both direct spray and dry residue tests. Direct spray tests were conducted on eggs and nymphs from the NY-1 colony, while dry residue tests were conducted with all three colonies and included eggs, nymphs, and adults. Mortality, oviposition, egg hatch, and development to adult were measured as response variables. Adults were also inspected and/or dissected under light microscope for morphological deformities or irregularities. All experiments were replicated four times.

The containers used to confine bed bugs during the course of each experiment with insecticide residues were prepared as follows: circular sections of Masonite (5 cm diameter) were sprayed with one of the treatments at indicated concentrations using a 118 ml fine mist spray bottle (SQB.4FMS, ProChemical and Dye, Somerset, MA). The spray bottles were triggered twice for each disk at a distance of approximately 15 cm, resulting in a uniformly wet surface. Application rates were calculated by weight over the treated

area. These treated disks were allowed to air-dry for 24 h, after which they were placed into the bottom of cylindrical plastic containers (42422, Consolidated Plastics, Stow, OH, radius 2.5 cm, depth 5 cm). The inside cylindrical surface was treated with Fluon® (polytetrafluorethylene, Northern Products, Woonsocket, RI), so that bugs placed inside were effectively confined to the bottom treated surface.

Each cell of a 24-well cell culture plate (3527, Corning Inc., Corning, NY) was lined at the bottom with filter paper, and the sides were coated with Fluon® to prevent escape. These cells were used to pair adults for mating following experimental treatments.

Over the course of these experiments, the following treatments were included: distilled water (control), (*S*)-methoprene 1x [diluted in distilled water to label concentration (0.007% methoprene)], (*S*)-methoprene 2x (0.014%), (*S*)-hydroprene 2x [diluted in distilled water to two times label concentration (0.12% hydroprene)], (*S*)-hydroprene 3x (0.18%), and (*S*)-hydroprene 10x (0.6%). The application method resulted in rates of 3.6 mg/m² and 7.4 mg/m² methoprene for (*S*)-methoprene 1x and 2x, respectively. Application rates for hydroprene were measured as 65.1 mg/m², 89.2 mg/m², and 317.8 mg/m² for (*S*)-hydroprene 2x, 3x, and 10x respectively.

Direct spray experiments

Eggs sprayed directly

Sections of blotter paper containing ten bed bug eggs (NY-1 colony) were directly sprayed with one of five treatments ((*S*)-methoprene (1x or 2x), (*S*)-hydroprene (2x or 3x), or control). Eggs had been deposited 0–24 or 48–72 h earlier. Each group of treated eggs was immediately transferred to an individual well of a 24-well cell culture plate.

Egg hatch was observed daily for eight days, at which point all eggs had either hatched or collapsed.

Nymphs sprayed directly

Third-instar nymphs from the NY-1 colony were directly sprayed with either (*S*)-hydroprene 3x or water. Each treatment group consisted of twenty nymphs, fed 24 h prior to treatment. Immediately following exposure, bugs were placed in untreated containers and kept in an incubator under the previously mentioned conditions. All bugs were fed weekly and observed for molting and mortality. Upon reaching the adult stage, each specimen was examined for morphological irregularities under a light microscope, then placed in an individual well on a 24-cell culture plate and fed. An untreated, fed virgin adult of the opposite gender was placed in each cell to allow mating. Subsequent oviposition, egg hatch and mortality were recorded for 20 d.

Dry Residue Experiments

Adults continuously exposed to dry residues

Masonite disks treated with (*S*)-methoprene (1x or 2x), (*S*)-hydroprene (2x or 3x) or water were placed in cylindrical containers. Groups of virgin bed bugs (five male and ten female) from the NY-1 colony were fed, placed in prepared containers on the dry, treated surface, and allowed to mate. The 1: 2 sex ratio minimized deleterious effects from multiple mating associated with traumatic insemination (Stutt and Siva-Jothy 2001). Mortality, oviposition, and egg hatch were observed daily for 20 d without additional feeding. At every observation, each bug was gently touched with clean forceps to determine responsiveness.

Nymphs continuously exposed to dry residues

Masonite disks treated with (*S*)-methoprene (1x or 2x), (*S*)-hydroprene (2x or 3x) or water were allowed to dry for 24 h. Groups of 20 third-instar nymphs from two colonies (NY-1 and FD) were fed, and then placed on the treated surfaces after 24 h. Nymphs were fed weekly and observed daily for molting and mortality. After reaching the adult stage, all insects were fed and placed in individual wells on a cell culture plate. An untreated virgin adult of the opposite gender was introduced in each cell to allow mating, and mortality, oviposition, and egg hatch were observed for 20 d. Every specimen was examined for morphological deformities using light microscope, and all females were dissected to count eggs remaining in the abdomen. Using these same methods, nymphs were also evaluated at higher concentration residues with treatments of (*S*)-hydroprene (3x or 10x) and a water control.

Nymphs from three colonies continuously exposed to dry residues

To examine potential efficacy against different colonies, three colonies were tested; NY-1, FD, and CIN-1. Masonite disks treated with (*S*)-hydroprene 3x or water were allowed to air dry for 24 h. Thirty fed and mated females were placed in each container and allowed to lay eggs for 24 h, after which all adults were removed. These eggs were allowed to hatch over the next two wks. Twenty first-instar nymphs from each container were randomly selected, fed, and returned to their respective treatment containers. Additional nymphs were discarded. These twenty nymphs were then followed through the remainder of their development to adults, with weekly feedings and observations of molting and mortality. Upon reaching the adult stage, they were placed in individual wells of a 24-well cell culture plate, fed and mated with untreated virgin

insects. Oviposition and egg hatch, along with mortality, were recorded for 20 d without additional feeding. At each observation, bugs were gently touched with clean forceps to determine responsiveness. In addition, each adult was examined under light microscope for morphological deformities. Containers were kept in an incubator between observations.

Statistical Analysis

Results were analyzed with Statistix 8.0 (Analytical Software, Tallahassee FL). Mean oviposition, egg hatch, successful development to adult, and retention of eggs were compared using Analysis of Variance. Proportions were arcsine transformed before analysis.

Results

Direct Spray Results

Direct spray of eggs

When eggs were sprayed directly, proportions of eggs that hatched were not statistically different for any treatments (water, (*S*)-methoprene 1x or 2x, (*S*)-hydroprene 2x or 3x), regardless of whether the eggs were sprayed at 1 or 3 d of development (F=0.87; df=4, 30; P=0.49). The hatch rates (mean ±SEM) were 0.98 ±0.025, 0.98 ±0.025, 1.0 ±0.0, 1.±0.0, and 0.93 ±0.048 for eggs sprayed when they were <24 h old with water, (*S*)-methoprene 1x, (*S*)-methoprene 2x, (*S*)-hydroprene 2x, or (*S*)-hydroprene 3x, respectively. For eggs treated when they were 48–72 h old, hatching rates were 1.0 ±0.0, 1.0 ±0.0, 0.95 ±0.05, 0.98 ±0.025, and 0.95 ±0.05 for the respective treatments.

Direct spray of nymphs

The mean proportion of nymphs successfully reaching the adult stage after being sprayed directly with (S)-hydroprene 3x was not significantly different than control (86.3% vs. 93.8%) (F=2.59; df=1, 6; P=0.16). Subsequent oviposition (mean eggs laid per female after first feeding as adult) was unaffected (F=0.49; df=1, 6; P=0.51) as was egg hatch (F=0.56; df=1, 6; P=0.48). Mean oviposition was 7.13 ±0.59 (SEM) and 6.5 ±0.69 for control and (*S*)-hydroprene 3x, respectively. Proportion hatching rates were 0.97 ±0.007 and 0.94 ±0.027 for the respective treatments. No morphological deformities were observed in the adults when examined under a light microscope.

Dry Residue Results

Adults continuously exposed to dry residues

For adults restricted to a treated surface, treatment effects were not significant for oviposition (F=0.2; df=4, 15; P=0.93) or egg hatch (F=0.49; df=4, 15; P=0.74). Mean oviposition (eggs laid per female after a single bloodmeal) was 6.05 ±0.58, 5.75 ±0.62, 5.55 ±0.86, 5.95 ±0.84, and 6.48 ±0.91 for water, (*S*)-methoprene 1x, (*S*)-methoprene 2x, (*S*)-hydroprene 2x, and (*S*)-hydroprene 3x treatments, respectively. These numbers are consistent with expectations for the first bout of oviposition after molting to adult. Proportional hatch rates were 0.90 ±0.021, 0.89 ±0.056, 0.94 ±0.006, 0.93 ±0.017, and 0.91 ±0.014 for the respective treatments.

Nymphs continuously exposed to dry residues

There was no effect on the proportion of treated nymphs developing to adult for any of the treatments (F=0.87; df=4, 30; P=0.49). For NY-1, the mean proportions developing to adults (n=4) were 0.88 ±0.043 (SEM), 0.90 ±0.054, 0.88 ±0.043, 0.91

±0.024, and 0.91 ±0.013 for water, (*S*)-methoprene 1x, (*S*)-methoprene 2x, (*S*)-hydroprene 2x, and (*S*)-hydroprene 3x, respectively. For FD colony, the mean proportions were 0.88 ±0.048, 0.93 ±0.025, 0.95 ±0.029, 0.89 ±0.032, and 0.85 ±0.02 for the respective treatments. Adults did show some other abnormalities associated with the treatments. There was a significant colony effect on oviposition (F=153.65; df=1, 30; P<0.0001) and on the number of eggs retained inside the female (F=43.75; df=1, 30; P<0.0001). Treatment by colony effect on oviposition was significant (F=3.28; df=4, 30; P=0.024); while there were no significant effects of treatments on the FD colony (F=1.39; df=4, 15; P=0.29), they were highly significant in the NY-1 colony (F=11.86; df=4, 15; P=0.0002), with adults resulting from NY-1 nymphs reared on a (*S*)-hydroprene 3x residue showing a 77% reduction in oviposition (Fig. 1A, B). Many of these females produced eggs but did not oviposit, instead retaining the developing eggs in the abdomen (Fig. 2A). Retained eggs did not hatch, but showed some development of the embryo, with eyes, legs, and distinct segmentation visible (Fig. 2B).

In an additional experiment, there was no significant difference in proportion of nymphs reaching adulthood for 3x, 10x, and control (F=1.6; df=2, 18; P=0.23; all treatments >0.88). Likewise, subsequent proportions of eggs hatching were high for all treatment (>0.85). There were strong treatment (F=36.8; df=2, 18; P<0.0001) colony (F=13.71; df=1, 18; P=0.0016), and treatment by colony (F=10.27; df=2, 18; P=0.0011) effects on the presence of morphological deformities. Morphological irregularities such as cuticular openings on the dorsal surface of the abdomen, color patchiness of the cuticle, malformed hemelytral pads, and in extreme cases, bursting of the gut through the dorsal surface of the abdomen at the site of cuticular openings were noted in different

proportions of NY-1 (0.09) and FD (0.05) bugs treated with (*S*)-hydroprene 3x, while control bugs showed no morphological deformities. Even more strikingly different proportions of NY-1 (0.81) and FD (0.23) bugs treated with (*S*)-hydroprene 10x exhibited these symptoms (Figs. 3, 4A). Oviposition also showed strong treatment (F=34.58; df=2, 18; P<0.0001), colony (F=116.9; df=1, 18; P<0.0001), and treatment by colony (F=6.27; df=2, 18; P=0.0086) effects. When treated with (*S*)-hydroprene 3x, NY-1 bugs showed decreases in oviposition similar to previous experiments (49% reduction), and no eggs were laid after rearing on (*S*)-hydroprene 10x. Oviposition of FD females showed a slight (13%) increase with (*S*)-hydroprene 3x, while decreasing (38%) with the (*S*)-hydroprene 10x treatment (Fig. 4B).

Nymphs from three colonies continuously exposed to dry residues

Adults resulting from treated nymphs from NY-1, FD and CIN-1 colonies exhibited results consistent with those of earlier experiments. There were no significant treatment effects on egg hatch (F=0.05; df=1, 18; P=0.83) or number reaching the adult stage (F=0.24; df=1, 18; P=0.63). Oviposition showed significant colony (F=14.44; df=2, 18; P=0.0002) and treatment (F=5.53; df=1, 18; P=0.03) effects. The number of eggs laid by (*S*)-hydroprene 3x treated bugs decreased by 40, 36, and 6% for NY-1, CIN-1, and FD respectively compared to the control (Fig. 5). No morphological defects were noted.

Discussion and conclusions

(*S*)-Methoprene had no effect on bed bugs at 1x and 2x label rate. These results are in agreement with Naylor and Boase (2008), who found significant results at 16 mg/m² (8.4 times label rate of Precor®) but not at 8 mg/m² (4.2 times label rate). Based

on these findings, methoprene does not appear to be a promising candidate for bed bug management programs.

The effects of (*S*)-hydroprene are dependent both on dose and bed bug strain, with two field strains more susceptible than the laboratory strain at 3X. Furthermore, oviposition was completely curtailed at the 10x rate for NY-1, and showed a 38% decrease in FD. The causal basis for the difference between laboratory and field strains is not clear.

Egg retention in females treated with hydroprene is an unusual effect; we are not aware of any similar published findings. Up to 36 developing eggs were found within a single female, while control females never contained more than two eggs. However, some treated females continued to lay eggs normally, and those hatched in normal proportions. For an individual female, either normal oviposition was displayed or all eggs were retained. While this condition could be due to various causes, it seems consistent with a morphological defect of the oviduct in which eggs cannot be deposited. However, crowding in the abdomen due to retained eggs left reproductive organs in poor condition, and dissections did not clearly show any specific defects.

Significant reduction in oviposition with (*S*)-hydroprene depended on prolonged exposure during nymphal instars, with nymphs feeding multiple times before effects were realized. Even after development to adult, bed bugs continued to feed despite decreased oviposition at the higher rates. The findings suggest that hydroprene has little if any immediate effect on bed bugs and the delayed effects occur only at rates above those on the current product label. Different effects might result when (*S*)-hydroprene is applied in

conjunction with other insecticides, as is often the case in commercial practice (Kramer et al. 1990, Moore and Miller 2008).

Based on our studies, current label rates of these JHA formulations would have little efficacy on bed bug populations. At application rates 3x label rate or higher, hydroprene would have a somewhat greater effect, but this would require label modification to allow higher rates of application and may not be feasible. However, given the limited availability of effective insecticides labeled for bed bugs, and the potential that some of these will be lost to regulation or resistance, further investigation of JHAs may be worthwhile.

Figure 5.1 A. Mean number (+SEM) of eggs laid by females from two colonies (NY-1, FD) reared on methoprene (M) or hydroprene (H) residues and mated with untreated virgin males; **B.** Mean number of eggs retained in the abdomen of treated females from the same colonies. For NY-1 colony, mean proportions of females retaining more than two eggs were 0.09, 0.19, 0.18, 0.38, and 0.82 for control, M 1x, M 2x, H 2x, and H 3x, respectively. For FD colony, corresponding mean proportions were 0, 0.02, 0, 0.08, and 0.04.

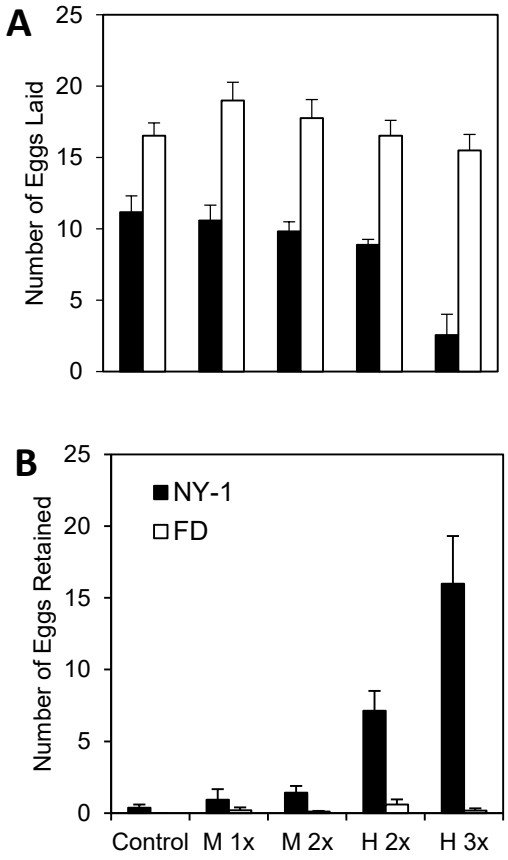


Figure 5.2 A. Dissected NY-1 female showing eggs retained in abdomen after treatment with (S)-hydroprene 3x; **B.** Close-up of egg retained in NY-1 female, showing development of embryo.

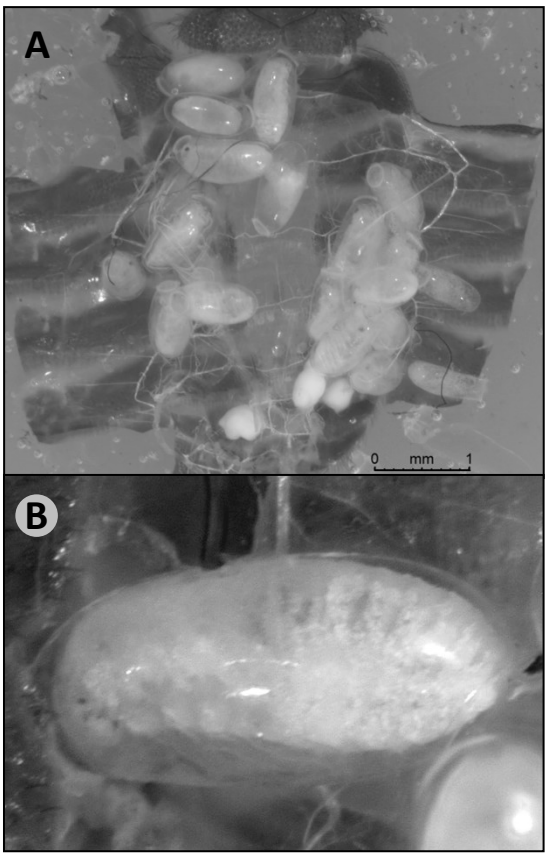


Figure 5.3 NY-1 adult showing morphological deformities; cuticular openings (co), abnormal hemelytral pads (ahp), and patchiness of cuticle (pc).

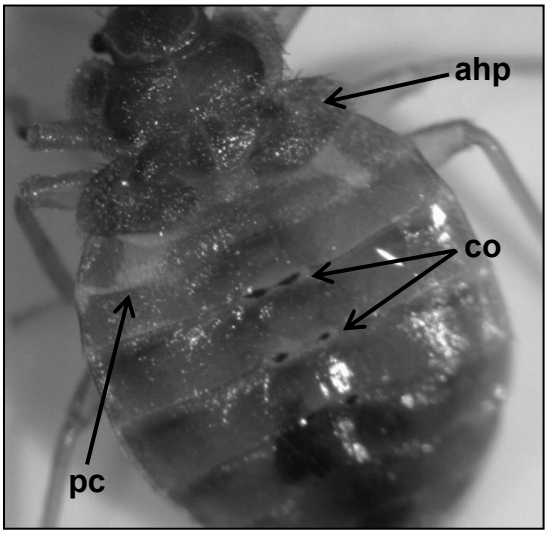


Figure 5.4 A. Mean proportion (+SEM) of adults that showed no morphological deformities from two colonies; **B.** Mean number of eggs produced by treated females from the same colonies paired with untreated virgin males.

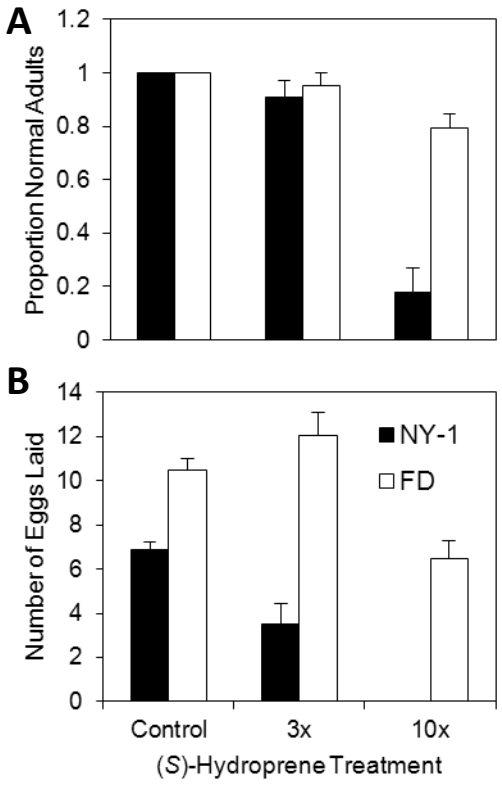
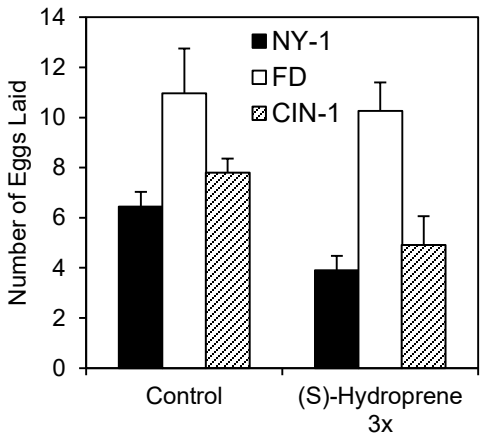


Figure 5.5 Mean number (+SEM) of eggs laid by treated females from three colonies.



Chapter 6: Conclusions

Since their resurgence at the turn of the century, bed bugs have become prominent in the public consciousness because of the economic, psychological and social impacts. While our overall understanding of bed bug biology and control has grown substantially over this time period, there has been relatively little research conducted on the endosymbiotic bacteria found within them. Researchers of other insect groups have powerfully demonstrated the critical role that symbionts play in the biology and ecology of their hosts, but we know little about the symbionts of bed bugs beyond their identity and some basic physical descriptions. In fact, early research on these microbes has been mostly descriptive in nature, with few attempts at experimentally elucidating the ways in which they affect their host. One key tool in studying these interactions is to eliminate symbionts in a subset of a population and compare these aposymbiotic bugs to a control group. My purpose was to determine the best methods for removing the endosymbionts of bed bugs and to discover more about bed bug endosymbiont interactions.

Two main approaches have been used to remove microorganisms from bed bugs for the purpose of studying symbiont-host interactions: rearing at increased temperatures (Chang 1974, Heaton 2013), and antibiotic treatment (Hosokawa et al. 2010, Heaton 2013, Nikoh et al. 2014). Both methods have positive and negative aspects affecting their suitability.

The use of temperature for symbiont elimination takes advantage of differences in thresholds for development and mortality between host and microbe. Bed bugs cease development at around 37°C and have a thermal death point between 44–45°C (Usinger 1966, Johnson 1941). There is a demonstrated decrease in symbiont titers when bugs are

reared at 36°C for two weeks (Chang 1974, Heaton 2013). However, Heaton (2013) also reports a subsequent symbiont titer rebound in the days following treatment, and my experiments with temperature yielded similar results. Rather than being killed outright, it seems probable that the symbionts here follow a pattern similar to that described by Mouton et al. (2005), in which densities of *Wolbachia* in aphid hosts decrease when they are maintained at increased temperatures.

In order to expand on this elimination technique, I extended treatment time from two to five weeks at 36°C. The resulting bed bug populations demonstrated increased mortality after five weeks, which placed an upper limit on the possible treatment time. The treatment also gave inconsistent results with regard to complete elimination of the symbionts; only a portion of replicate strains continued to show low symbiont titers over time. The rebound in symbiont titers following rearing at elevated temperature is a problem for subsequent experimentation, since it is difficult to differentiate the effects of temperature versus symbiont loss on the host as endosymbiont titers recover to original levels. I concluded that rearing the host at elevated temperatures yielded results too inconsistent and unstable for reliable use in experiments.

The use of antibiotics can also be complicated, with direct impacts on the host easily confounded with effects of symbiont loss. It has been suggested that incorporating multiple antibiotic treatments with different modes of action as controls is unsatisfactory because each antibiotic will have unknown effects (Heaton 2013). However, if different antibiotics each result in removal of the symbionts but have different impacts on the host, the treatments can be contrasted to help differentiate between those impacts occurring due to symbiont loss and those caused by the treatment directly.

For example, my experiments with four different antibiotics showed that at least some of the host effects documented with tetracycline-type antibiotics, including reduced fecundity (Heaton 2013), may be attributed to the toxicity of antibiotics to bed bugs and not to symbiont loss, since the loss of symbionts with rifamycin treatment does not result in the same immediate fecundity reduction. These results are notable because tetracycline is often used to remove symbionts in other systems (Min and Benzer 1997, Puttaraju and Prakash 2005, Dobson and Rattanadechakul 2001). Potential complications with tetracycline toxicity could be associated with binding to blood serum such as would take place in blood fed to bed bugs (Rolinson and Sutherland 1965) and toxic breakdown products (Benitz and Diermeier 1964). As I have shown that it has detrimental effects directly on the bed bug host, it may have detrimental direct effects on other insects as well. Tetracyclines have also recently been shown to impair the function of mitochondria in eukaryotes, with potentially confounding influence on research looking at the effects of symbiont loss (Ballard and Melvin 2007, Moullan et al. 2015, Chatzispyrou et al. 2015). This is not the first example of sometimes long-term non-target effects of tetracycline used for the purpose of removing symbionts. For example, tetracycline treatment reduced sperm viability in male pseudoscorpions, but also caused reduced sperm viability in the sons of treated males (Zeh et al. 2012), though the impact did not extend to the second generation. Tetracycline was also shown to cause reduced offspring and male-skewed gender bias in *Drosophila*, independent of symbiont effects (O'Shea and Singh 2015). This is notable because these very effects have been attributed to symbionts in some insects (Werren et al. 2008).

The results of my research on methods of symbiont removal show a simple yet effective way to eliminate these microorganisms in bed bugs and create aposymbiotic strains for experimental comparisons by feeding adults weekly with blood containing rifamycin. After three weeks of treatment, symbiont titers are reduced sufficiently that offspring of these treated adults can be collected and used to initiate consistently aposymbiotic strains. Although there are some reports of permanent and heritable effects of antibiotics on insects, such as changes in mitochondrial metabolism (Ballard and Melvin 2007, Zeh et al. 2012), using the offspring of treated bugs for experiments rather than using those that are directly treated with antibiotics does reduce the likelihood of confounding results due to antibiotic effects on the host.

While in my studies the permanent establishment of aposymbiotic strains was hampered by increasingly long development times in successive generations, some researchers have published evidence that aposymbiotic bed bugs can regain nearly normal fecundity and development rate through supplementation of their diets with specific B vitamins (Hosokawa et al. 2010, Nikoh et al. 2014, Heaton 2013). In fact, Nikoh et al. (2014) showed that the genome of *Wolbachia* in bed bugs includes the entire genetic pathway for synthesizing biotin and riboflavin. My experiments, conducted prior to this discovery, provided vitamin supplements to aposymbiotic bugs including riboflavin and several other B vitamins, but I did not include biotin in my tests. However, Nikoh et al. (2014) also presented results using the same mix of vitamins I used, and the improvement of host fecundity and development in their trials was much greater than in mine. The difference in results has several possible explanations, such as variations in rearing conditions, feeding methods, the bed bug strains examined, or the use of bugs

which originally only contained *Wolbachia*, as opposed to populations which originally harbored both symbionts. It is also possible that my use of later generations of aposymbiotic bugs for the experiment, as opposed to treated bugs, played a part. Regardless, the use of this method (offspring of bugs treated with antibiotics, fed on supplemented blood) opens the door for future studies on symbiont impacts.

The decreased size of aposymbiotic bed bugs demonstrated in our studies may be attributed to malnutrition from lack of biotin and other nutrients. Similar results were published by Kuriwada et al. (2010) following the removal of the symbiont *Nardonella* in a weevil, with reduced host size continuing into subsequent generations. It is also likely that there are other interactions with the symbionts of which we are not aware and have not considered. Substantial variation in bed bug size within colonies has long been observed in our laboratories and in the field, but there has not been any attempt to correlate the size of naturally-occurring bed bugs with presence of symbionts, and the results may be informative.

A notable finding of this work is the horizontal transmission of BLS, but not *Wolbachia*, from male bed bugs to females via traumatic insemination. While horizontal transmission of facultative endosymbionts such as *Regiella* during sexual reproduction is documented in aphids, (Moran and Dunbar 2006), this is the first example of horizontal transmission in bed bugs, and the first demonstrated for any insect through traumatic insemination. This new information on how symbionts may be maintained in a population could be useful in future studies on the impacts of BLS on the bed bug host. If the bacteria can be easily and naturally reintroduced to an aposymbiotic bed bug strain through mating, it could help clarify possible confounding antibiotic effects and complete

Koch's postulates to prove the effects of BLS loss. It could also aid in differentiating between effects caused by the loss of the two different endosymbionts. My experiments measured for but did not demonstrate that BLS is subsequently transmitted to offspring after horizontal transmission from a male, but this would be another unusual finding. Generally, symbionts are transmitted from mother to offspring, but transmission from both parents to offspring would provide an intriguing contrast and interaction with the maternally inherited *Wolbachia*.

In my transmission study, over half of the aposymbiotic females mating with a single normal male tested positive for BLS after just four weeks. As indicated by Stutt and Siva-Jothy (2001), a male bed bug will mate with multiple females within a population after each blood meal. In a normal population, this would likely result in few if any females that have not mated with BLS-positive males.

One might expect higher rates of infection in females, therefore, since horizontal infection only occurs one direction, but this does not seem to be the case. I found that within the NY1 population about 10% of virgin adult individuals, both male and female, lacked BLS. This proportion remained constant over the course of several years, and appears stable within the population. It appears reasonable that while some populations are truly free of BLS due to founder effects, few if any are completely made up of infected individuals. Further studies must be done to determine the efficiency of BLS transmission, both transovarially and during insemination. It also remains to be determined if transmission to females during mating results in transmission to offspring as well, or only to the female.

There was an initial fecundity benefit for aposymbiotic virgin females if mated with a male having symbionts, as compared to a male lacking symbionts. This finding is in agreement with the work of Heaton (2013), and begs the question of whether the benefit is tied to the transmission of BLS to the female, male viability, or nutrients provided to the female by a symbiont-infected male. One study showed that increased amounts of ejaculate increased the fecundity of female bed bugs regardless of mating rate (Reinhardt et al. 2009). Because aposymbiotic males are significantly smaller than normal males, the fecundity benefit may simply be a reflection of adult size difference impacting amounts of ejaculate available for mating. Over time, multiple mating events might then result in a narrowing of the resulting fecundity gap. This hypothesis remains to be tested. On the other hand, considering that a single mating event may allow a female bed bug to lay viable eggs for eight weeks or more (Johnson 1941), it is possible that other ejaculate components, and not insufficient sperm, are the cause of reduced fecundity.

Control of bed bugs as a human pest is an ongoing concern, which leads to the question of whether endosymbiont effects on their host may indicate a weakness we could exploit in bed bug management, or even be somehow responsible for difficulty in their control. It has been demonstrated that in some stink bugs, insecticide resistance is conferred by symbionts (Kikuchi et al. 2012). However, I tested both resistant and susceptible populations of bed bugs and found *Wolbachia* and BLS present in all of them, indicating that insecticide resistance in bed bugs is unlikely to be related to symbiont effects. On the other hand, if bed bug populations are dependent on the nutrients provided by symbionts for successful development and fecundity, it is tempting to suggest

elimination of symbionts as a control method. This is not reasonable, because even if a proper feeding mechanism could be contrived which would provide the majority of bugs in an infestation with multiple blood meals, it would still be simpler to feed the bugs something to kill them outright rather than attempting to lower their reproductive potential and growth over several months.

Control of a pest through integrated pest management hinges in great part on a correct understanding of its biology, ecology, and behavior. Microbial endosymbionts have dramatic impact on their hosts, and the bed bug is no exception. Thus as *C. lectularius* becomes ever more prevalent as a pest, our understanding of these interactions increases in importance, and further research is needed to answer questions which have not been sufficiently addressed. For example, do symbiont titers have thresholds to affect function in the host? What are the interactions between *Wolbachia* and BLS in bed bugs? Are there changes is natural behavior due to symbiont loss, such as feeding frequency, defecation, and mating behaviors? Does symbiont loss impact bed bug effectiveness as a disease vector? In addition, learning about the symbionts of the bed bug sheds light on possible roles of other symbionts in insects, both related and unrelated. As research in this area continues, new applications and possibilities will undoubtedly emerge.

REFERENCES

Adelman, Z. N., D. M. Miller, and K. M. Myles. 2013. Bed bugs and infectious disease: a case for the arboviruses. PLoS Pathog 9(8): e1003462. doi:10.1371/journal.ppat.1003462.

Ahantarig, A., W. Trinachartvanit, and P. Kittayapong. 2008. Relative *Wolbachia* density of field-collected *Aedes albopictus* mosquitoes in Thailand. J Vector Ecol 33: 173–177.

Akhoundi, M., A. Cannet, C. Loubatier, J. Berenger, A. Izri, P. Marty, and P. Delaunay. 2016. Molecular characterization of *Wolbachia* infection in bed bugs (*Cimex lectularius*) collected from several localities in France. Parasite 23(31) DOI: 10.1051/parasite/2016031

Andrews, E. S. 2013. Analyzing arthropods for the presence of bacteria. Curr Protocols Microbiol 28: E:1E.6:1E.6.1–1E.6.14.

Arkwright, J. A., E. E. Atkin, and A. Bacot. 1921. An hereditary Rickettsia-like parasite of the bed bug (*Cimex lectularius*). Parasitology 13: 27–38.

Aschner, M. 1934. Studies on the symbiosis of the body louse. I. Elimination of the symbionts by centrifugation of the eggs. Parasitology 26: 309–314.

Ashcroft, R., Y. Seko, L. F. Chan, J. Dere, J. Kim, and K. McKenzie. 2015. The mental health impact of bed bug infestations: a scoping review. Int J Public Health 60(7): 827–837. doi: 10.1007/s00038-015-0713-8.

Baines, S. 1956. The role of the symbiotic bacteria in the nutrition of *Rhodnius prolixus* (Hemiptera). J Exp Biol 33: 533–541.

Ballard, J. W. O., and R. G. Melvin. 2007. Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in *Drosophila*. Insect Mol Biol 16: 799–802.

Bandi, C., A. J. Trees, and N. W. Brattig. 2001. *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. Vet Parasitol 98: 215–238.

Barrozo, R. B., S. A. Minoli, and C. R. Lazzari. 2004. Circadian rhythm of behavioural responsiveness to carbon dioxide in the blood-sucking bug *Triatoma infestans* (Heteroptera: Reduviidae). J Insect Physiol 50: 249–254.

Bartley, J. D., and H. J. Harlan. 1974. Bed bug infestation: its control and management. Mil Med 139: 884–886.

Beard, C. B., C. Cordon-Rosales, and R. V. Dursavasula. 2002. Bacterial symbionts of the Triatominae and their potential use in control of Chagas Disease transmission. Annu Rev Entomol 47: 123–141.

Beard, C. B., R. V. Dursavasula, and F. F. Richards. 1998. Bacterial symbiosis in arthropods and the control of disease transmission. Emerg Infect Dis 4: 581–591.

Benitz, K. F., and H. F. Diermeier. 1964. Renal toxicity of tetracycline degradation products. Proc Soc Exp Biol Med 115: 930.

Benoit, J. B., N. A. Del Grosso, J. A. Yoder, and D. L. Denlinger. 2007. Resistance to dehydration between bouts of blood feeding in the bed bug, *Cimex lectularius*, is enhanced by water conservation, aggregation, and quiescence. Am J Trop Med Hyg 76(5): 987–993.

Benoit, J. B., A. J. Jajack, and J. A. Yoder. 2012. Multiple traumatic insemination events reduce the ability of bed bug females to maintain water balance. J Comp Physiol B 182(2): 189–198.

Benoit, J. B., G. Lopez-Martinez, N. M. Teets, S. A. Phillips, and D. L. Denlinger. 2009. Responses of the bed bug, *Cimex lectularius*, to temperature extremes and dehydration: levels of tolerance, rapid cold hardening and expression of heat shock proteins. Med Vet Entomol 23(4): 418–425.

Berlese, A. 1898a. Fenomeni che accompagnano la fecondazione in taluni insetti I. Riv Patol Veg 6(3): 1-16.

Berlese, A. 1898b. Fenomeni che accompagnano la fecondazione in taluni insetti II. Riv Patol Veg 7(1): 1-18.

Blow, J. A., M. J. Turell, A. L. Silverman, and E. D. Walker. 2001. Stercorarial shedding and transtadial transmission of hepatitis B virus by common bed bugs (Hemiptera: Cimicidae). J Med Entomol 38: 694–700.

Boase, C. 2009. Bedbugs - information upsurge. Internatl Pest Cont 54(4): 181–184.

Bourtzis, K., and S. O'Neill. 1998. *Wolbachia* infections and arthropod reproduction. BioScience 48: 287–293.

Brecher, G., and V. B. Wigglesworth. 1944. The transmission of *Actinomyces rhodnii* Erikson in *Rhodnius prolixus* stål (Hemiptera) and its influence on the growth of the host. Parasitology 35(4): 220–224.

Bright, M., and S. Bulgheresi. 2010. A complex journey: transmission of microbial symbionts. Nature Rev Microbiol 8: 218–230.

Brooks, M. A. 1964. Symbiotes and the nutrition of medically important insects. B World Health Organ 31: 555–559.

Brooks, M. A., and A. G. Richards. 1955. Intracellular symbiosis in cockroaches .1. Production of aposymbiotic cockroaches. Biol Bull 109: 22–39.

Brownlie, J. C., and K. N. Johnson. 2009. Symbiont-mediated protection in insect hosts. Trends Microbiol 17(8): 348–354.

Brownlie, J. C., B. N. Cass, M. Reigler, J. J. Witsenburg, I. Iturbe-Ormaetxe, E. A. McGraw, and S. L. O'Neill. 2009. Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. PLoS Pathog. 5(4): e1000368. doi: 10.1371/journal.ppat.1000368.

Brumin, M., M. Levy, and M. Ghanim. 2012. Transovarial transmission of Rickettsia spp. And organ-specific infection of the whitefly *Bemisia tabaci*. Appl Environ Microbiol 78(16): 5565–5574.

Buchner, P. 1921. Uber ein neues symbiontisches organ der Bettwanze. A new symbiotic organ of the bed bug. Biologisches Zentralblatt 41(12): 570–574.

Buchner, P. 1923. Studien an intracellularen Symbionten. IV. Die Bakteriensymbiose der Bettwanze. Arch Protistenkd 46: 225–263.

Butler, K., A. R. English, V. A. Ray, and A. E. Timreck. 1970. Carbenicillin: chemistry and mode of action. J Infect Dis 122: S1-S8.

Campbell, B.C., and A. H. Purcell. 1993. Phylogenetic affiliation of BEV, a bacterial parasite of the leafhopper *Euscelidius variegatus*, on the basis of 16S rDNA sequences. Curr Microbiol 26: 37–41.

Chang, K. P. 1974. Effects of elevated temperature on the mycetome and symbiotes of the bed bug *Cimex lectularius* (Heteroptera). J Invertebr Pathol 23: 333–340.

Chang, K. P. 1975. Haematophagous insect and haemoflagellate as hosts for prokaryotic endosymbionts. Sym Soc Exp Biol 29: 407–428.

Chang, K. P., and A. J. Musgrave. 1973. Morphology, histochemistry, and ultrastructure of mycetome and its rickettsial symbiotes in *Cimex lectularius* L. Can J Microbiol 19(9): 1075–1081.

Chatzispyrou, I. A., N. M. Held, L. Mouchiroud, J. Auwerx, and R. H. Houtkooper. 2015. Tetracycline antibiotics impair mitochondrial function and its experimental use confounds research. Cancer Res 75(21): 4446–4449.

Cleveland, L. R. 1925. Toxicity of oxygen for protozoa in vivo and in vitro: animals defaunated without injury. Biol Bull 48: 455.

Cleveland, L. R., and A. W. Burke, Jr. 1956. Effects of temperature and tension on oxygen toxicity for the protozoa of *Cryptocercus*. J Protozool 3: 74–77.

Cooper, R., C. Wang, and N. Singh. 2015. Mark-release-recapture reveals extensive movement of bed bugs (*Cimex lectularius* L.) within and between apartments. Plos One 10(9): e0136462.

Dasgupta, B., and H. N. Ray. 1955. Studies on the follicle cells of the bed bug. P Zool Soc Calcutta 8: 1–7.

Dasgupta, B., and H. N. Ray. 1956. Observations on the corpus luteum and the NR bodies in the female gonads of bed bug. P Zool Soc Calcutta 9: 55–63.

de Crespigny, F. E. C., and N. Wedell. 2006. *Wolbachia* infection reduces sperm competitive ability in an insect. P Roy Soc Lond B Bio 273: 1455–1458.

de Crespigny, F. E. C., T. D. Pitt, and N. Wedell. 2006. Increased male mating rate in *Drosophila* is associated with *Wolbachia* infection. J Evolution Biol 19: 1964–1972.

De Meillon, B., and L. Goldberg. 1946. Preliminary studies on the nutritional requirements of the bed bug (*Cimex lectularius* L.) and the tick *Ornithodorus moubata* Murray. J Exp Biol 24: 41–61.

De Meillon, B., J. M. Thorpe, and F. Hardy. 1947. The development of *Cimex lectularius* and *Ornithodorus moubata* on riboflavin deficient rats. S Afr J Med Sci 12: 111–116.

Dedeine, F., F. Vavre, F. Fleury, B. Loppin, M. E. Hochberg, and M. Bouletreau. 2001. Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. P Natl Acad Sci USA 98: 6247–6252.

Dobson, S. L., and W. Rattanadechakul. 2001. A novel technique for removing *Wolbachia* infections from *Aedes albopictus* (Diptera: Culicidae). J Med Entomol 38: 844–849.

Douglas, A. E. 1989. Mycetocyte symbiosis in insects. Biol Rev 69: 409–434.

Eichler, S., and G. A. Schaub. 1998. The effects of aposymbiosis and of an infection with *Blastocrithidia triatomae* (Trypanosomatidae) on the tracheal system of the Reduviid bugs *Rhodnius prolixus* and *Triatoma infestans*. J Insect Physiol 44(2): 131–140.

Fenollar, F., M. Maurin, and D. Raoult. 2003. *Wolbachia pipientis* growth kinetics and susceptibilities to 13 antibiotics determined by immunofluorescence staining and real-time PCR. Antimicrob Agents Chemother 47(5): 1665-1671.

Floss, H. G., and T. W. Yu. 2005. Rifamycin—mode of action, resistance, and biosynthesis. Chem Rev 105(2): 621–632.

Goddard, J. 2003. Bed bugs bounce back—But do they transmit disease? Infect Med 20: 473–474.

Goddard, J., and R. deShazo. 2009. Bed bugs (*Cimex lectularius*) and clinical consequences of their bites. J Amer Med Assn 301(13): 1358–1366.

Goodman, M. H., M. F. Potter, and K. F. Haynes. 2013. Effects of juvenile hormone analog formulations on development and reproduction in the bed bug Cimex lectularius (Hemiptera: Cimicidae). Pest Manag Sci 69(2): 240–244.

Gordon, J. R., M. H. Goodman, M. F. Potter, and K. F. Haynes. 2014. Population variation in and selection for resistance to pyrethroid-neonicotinoid insecticides in the bed bug. Sci Rep 4(3836): 1–7.

Guruprasad, N. M., L. Mouton, and H. P. Puttaraju. 2011. Effect of *Wolbachia* infection and temperature variations on the fecundity of the Uzifly *Exorista sorbillans* (Diptera: Tachinidae). Symbiosis 54: 151. doi:10.1007/s13199-011-0138-y

Haine, E. R. 2007. Symbiont-mediated protection. Proc Biol Sci. 275(1633): 353–361.

Harraca, V., C. Ryne, and R. Ignell. 2010b. Nymphs of the common bed bug (*Cimex lectularius*) produce anti-aphrodisiac defence against conspecific males. BMC Biol 8: 121–

Harraca, V., R. Ignell, C. Lofstedt, and C. Ryne. 2010a. Characterization of the antennal olfactory system of the bed bug (*Cimex lectularius*). Chem Senses 35(3): 195–204.

Harrington, J. S. 1960. Studies on *Rhodnius prolixus*: growth and development of normal and sterile bugs, and the symbiotic relationship. Parasitology 50: 279–286.

Hase, A. 1930. Weitere versuche zur kenntnis der bettwanzen *Cimex lectularius* L. und *Cimex rotundatus* Sign. (Hex.-Rhynch.). Parasitol Res 2: 368–418.

Heaton, L.L. 2013. *Wolbachia* in bed bugs *Cimex lectularius*. PhD Thesis, University of Sheffield, Sheffield.

Henrick, C. A. 2007. Methoprene. J Am Mosq Control Assoc 23(supplement): 225–239.

Henry, T. J. 2009. Biodiversity of Heteroptera, pp. 223–263. *In* Insect Biodiversity: Science and Society. R. G. Foottit, P. H. Adler (eds.), Blackwell Publishing, Oxford.

Hermans, P. G., C. A. Hart, and A. J. Trees. 2001. In vitro activity of antimicrobial agents against the endosymbiont *Wolbachia pipientis*. J Antimicrob Chemother 47(5): 659-63.

Hertig, M., and S. B. Wolbach. 1924. Studies on Rickettsia-like microorganisms in insects. J Med Res 44: 329–374.

Higgins, J. A., and A. F. Azad. 1995. Use of polymerase chain reaction to detect bacteria in arthropods: a review. J Med Entomol 32: 213–222.

Honěk, A. 1993. Intraspecific variation in body size and fecundity in insects: a general relationship. OIKOS 66: 483–492.

Hosokawa, T., R. Koga, Y. Kikuchi, X. Y. Meng, and T. Fukatsu. 2010. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. P Natl Acad Sci USA 107: 769–774.

Huigens, M. E., R. P. de Almeida, P. A. H. Boons, R. F. Luck, and R. Stouthamer. 2004. Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. P Roy Biol Sci Lond B 271: 509–515.

Hurst, G. D. D., A. P. Johnson, J. H. G. Schulenburg, and Y. Fuyama. 2000. Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. Genetics 156: 699–709.

Hypsa, V., and S. Aksoy. 1997. Phylogenetic characterization of two transovarially transmitted endosymbionts of the bedbug *Cimex lectularius* (Heteroptera: Cimicidae). Insect Mol Biol 6: 301–304.

Johnson, C. G. 1937. The relative values of man, mouse, and domestic fowl as experimental hosts for the bed-bug, *Cimex lectularius*. P Zool Soc Lond A 107: 107–126.

Johnson, C. G. 1941. The ecology of the bed bug, *Cimex lectularius* L., in Britain: report on research, 1935–40. J Hyg-Cambridge 41: 345–461.

Kikuchi, Y., M. Hayatsu, T. Hosokawa, A. Nagayama, K. Tago, and T. Fukatsu. 2012. Symbiont-mediated insecticide resistance. P Natl Acad Sci USA 109(22): 8618–8622.

Knell, R. J., and K. M. Webberley. 2004. Sexually transmitted diseases of insects: distribution, evolution, ecology and host behavior. Biol Rev 79(3): 557–581.

Koch, A. 1960. Intracellular symbiosis in insects. Annu Rev Microbiol 14: 121–140.

Koehler, P. G. 2001. Bed bugs are back. Pest Control Technology 29(9): 80–81.

Koga, R., T. Tsuchida, and T. Fukatsu. 2003. Changing partners in an obligate symbiosis: A facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. Proc R Soc London Ser B 270: 2543–2550.

Koga, R., T. Tsuchida, M. Sakurai, and T. Fukatsu. 2007. Selective elimination of aphid symbionts: effects of antibiotic dose and host genotype, and fitness consequences. FEMS Microbiol Ecol 60: 229–239.

Kramer, R. D., P. G. Koehler, and J. S. Patterson. 1990. Effects of hydroprene exposure on the physiology and insecticide susceptibility of German cockroaches (Orthoptera: Blattellidae). J Econ Entomol 83: 2310–2316.

Krieg, A. 1959. Über die Natur von NR-bodies" bei Rickettsien-Infektionen von Insekten. Naturwissenshaften 46: 231–232.

Kuriwada, T., Hosokawa, T., Kumano, N., Shiromoto, K., Haraguchi, D., and Fukatsu, T. 2010. Biological role of *Nardonella* endosymbiont in its weevil host. PLoS ONE, 5(10), e13101. http: //doi.org/10.1371/journal.pone.0013101

Lake, P., and W. G. Friend. 1968. The use of artificial diets to determine some of the effects of *Nocardia rhodnii* on the development of *Rhodnius prolixus*. J Insect Physiol 14(4): 543–62.

Leonardo, T. E., and G. T. Muiru. 2003. Facultative symbionts are associated with host plant specialization in pea aphid populations. Proc Biol Sci 270(suppl 2): S209–212.

Louis, C., M. Laporte, J. Carayon, and C. Vago. 1973. Mobilité, ciliature et caractères ultrastructuraux des micro-organismes symbiotiques endo et exocellulaires de

Cimex lectularius L. (Hemiptera Cimicidae). CR Acad Sci III Vie 277(6): 607–611.

Liu, F., K. F. Haynes, A. G. Appel, and N. Liu. 2014. Antennal olfactory sensilla responses to insect chemical repellents in the common bed bug, *Cimex lectularius*. J Chem Ecol 40(6): 522–533.

Mavale, M., D. Parashar, A. Sudeep, M. Gokhale, Y. Ghodke, G. Geevarghese, V. Arankalle, and A. C. Mishra. 2010. Venereal Transmission of Chikungunya Virus by *Aedes aegypti* Mosquitoes (Diptera: Culicidae). Am J Trop Med Hyg 83(6): 1242–1244. http://doi.org/10.4269/ajtmh.2010.09-0577

McAllister, T. A., J. M. Stark, J. N. Norman, and R. M. Ross. 1964. Inhibitory effects of hyperbaric oxygen on bacteria and fungi. Lancet 2: 1040–1042.

Mee, P. T., A. R. Weeks, P. J. Walker, A. A. Hoffmann, and J. B. Duchemin. 2015. Detection of low-level *Cardinium* and *Wolbachia* infections in *Culicoides*. Appl Environ Microbiol 81(18): 6177–6188.

Meriweather, M., S. Matthews, R. Rio, and R. S. Baucom. 2013. A 454 survey reveals the community composition and core microbiome of the common bed bug (*Cimex lectularius*) across an urban landscape. PloS one 8(4): e61465.

Min, K. T., and S. Benzer. 1997. *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. P Natl Acad Sci USA 94(20), 10792–10796.

Mohandass, S. M., F. H. Arthur, K. Y. Zhua, and J. E. Throne. 2006. Hydroprene: mode of action, current status in stored-product pest management, insect resistance, and future prospects. Crop Prot 25: 902–909.

Montes, C., C. Cuadrillero, and D. Vilella. 2002. Maintenance of a laboratory colony of *Cimex lectularius* (Hemiptera: Cimicidae) using an artificial feeding technique. J Med Entomol 39: 675–679.

Moore, D. J., and D. M. Miller. 2008. Field evaluations of insecticide treatment regime control of the common bedbug, *Cimex lectularius* (L.). Pest Manag Sci 65: 332–338.

Moran, N. A., and H. E. Dunbar. 2006. Sexual acquisition of beneficial symbionts in aphids. P Natl Acad Sci USA 103: 12803–12806.

Moran, N. A., J. P. McCutcheon, and A. Nakabachi. 2008. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet 42: 165–90.

Morrow, E. H., and G. Arnqvist. 2003. Costly traumatic insemination and a female counter-adaptation in bed bugs. P Roy Soc Lond B 270: 2377–2381.

Moullan, N., L. Mouchiroud, X. Wang, D. Ryu, E. G. Williams, A. Mottis, V. Jovaisaite, M. V. Frochaux, P. M. Quiros, B. Deplancke, R. H. Houtkooper, and J. Auwerx. 2015. Tetracyclines disturb mitochondrial function across eukaryotic models: a call for caution in biomedical research. Cell Reports 10(10): 1681-1691.

Mouton, L., H. Henri, M. Bouletreau, and F. Vavre. 2006. Effect of temperature on *Wolbachia* density and impact on cytoplasmic incompatibility. Parasitology 132: 49–56. doi: 10.1017/S0031182005008723.

Müller, M. J., N. C. D. Dörr, M. Deprá, H. J. Schmitz, V. H. Valiati, and V. L. da Silva Valente. 2013. Reevaluating the infection status by the *Wolbachia*

endosymbiont in *Drosophila* Neotropical species from the *willistoni* subgroup. Infect Gen Evol 19: 232–239.

Nakabachi, A., and H. Ishikawa. 1999. Provision of riboflavin to the host aphid, *Acyrthosiphon pisum*, by endosymbiotic bacteria, *Buchnera*. J Insect Physiol. 45(1): 1–6.

Naylor, R. 2012. Ecology and dispersal of the bed bug. PhD Thesis, University of Sheffield, Sheffield.

Naylor, R., D. Bajomi, and C. Boase. 2008. Efficacy of (S)-methoprene against *Cimex lectularius* (Hemiptera: Cimicidae), pp. 115–121. *In* W. H. Robinson and D. Bajomi (eds.), Proceedings, Sixth International Conference on Urban Pests, 13–16 July 2008, Budapest, Hungary. OOK-Press Kft, Budapest, Hungary.

Nikoh, N., T. Hosokawa, M. Moriyama, K Oshima, M. Hattori, and T. Fukatsu. 2014. Evolutionary origin of insect–*Wolbachia* nutritional mutualism. P Natl Acad Sci USA 111(28): 10257–10262.

Novelli, V. M., J. Freitas-Astua, N. Segatti, J. L. C. Mineiro, V. Arthur, M. Bastianel, M. E. Hilf, T. R. Gottwald, and M. A. Machado. 2008. Effects of radiation (Cobalt-60) on the elimination of *Brevipalpus phoenicis* (Acari: Tenuipalpidae) *Cardinium* endosymbiont. Exp Appl Acarol 45: 147–153.

O'Shea, K. L., and N. D. Singh. 2015. Tetracycline-exposed *Drosophila melanogaster* males produce fewer offspring but a relative excess of sons. Ecol Evol, 5(15), 3130–3139. http: //doi.org/10.1002/ece3.1535

Oliver, K. M., J. A. Russell, N. A. Moran, and M. S. Hunter. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. P Natl Acad Sci USA 100(4): 1803–1807.

Olson, J. F., R. D. Moon, and S. A. Kells. 2009. Off-host aggregation behavior and sensory basis of arrestment by *Cimex lectularius* (Heteroptera: Cimicidae). J Insect Physiol. 55(6): 580–587.

Otti, O., R. A. Naylor, M. T. Siva-Jothy, and K. Reinhardt. 2009. Bacteriolytic activity in the ejaculate of an insect. Am Nat 174(2): 292–295.

Oulhen, N., B. J. Schulz, and T. J. Carrier. 2016. English translation of Heinrich Anton de Bary's 1878 speech, 'Die Erscheinung der Symbiose' ('De la symbiose'). Symbiosis 69: 131. doi:10.1007/s13199-016-0409-8

Pais, R., C. Lohs, Y. Wu, J. Wang, and S. Aksoy. 2008. The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. Appl Environ Microbiol 74(19): 5965–5974.

Parashar, B. D., K. Ganesan, D. Sukumaran, Y. V. S. Rao, V. Vijay, and S. Prakash. 2003. Aggregation activity induced by the excreta extracts in *Cimex hemipterus* (Hemiptera: Cimicidae). Entomon 28(3): 215–222.

Patton, W. S., and F. W. Cragg. 1913. Cimicidae, pp. 479–524. *In* A Textbook of Medical Entomology. Christian Lit. Soc. for India, London.

Perotti, M. A., J. M. Allen, D. L. Reed, and H. R. Braig. 2007. Host-symbiont interactions of the primary endosymbiont of human head and body lice. FASEB J 21: 1058–1066.

Pfeiffer, H. 1931. Beitrage zu der bakteriensymbiose der bettwanze (*Cimex lectularius*) und der Schwalbenwanze (*Oeciacus hirundinis*). Zbl Bakt II Natur 123: 151–171.

Pfiester, M., P. Koehler, and R. Pereira. 2009a. Effect of population structure and size on aggregation behavior of *Cimex lectularius* (Hemiptera: Cimicidae). J Med Entomol 46(5): 1015–1020.

Pfiester, M., P. G. Koehler, and R. M. Pereira. 2009b. Sexual conflict to the extreme: traumatic insemination in bed bugs. Am Entomol 55(4): 244–249.

Philip, C. B. 1956. Comments on the classification of the order Rickettsiales. Can J Microbiol 2: 261–270.

Polanco, A. M., C. C. Brewster, and D. M. Miller. 2011. Population growth potential of the bed bug, *Cimex lectularius* L.: a life table analysis. Insects 2(2): 173–185.

Potter, M. F. 2006. The perfect storm: an extension view on bed bugs. Am Entomol 52: 102–104.

Potter, M. F. 2008. Bed bug supplement: the history of bed bug management. Pest Control Technology 36: S1.

Potter, M. F., K. F. Haynes, and J. Fredericks. 2015. Bed bugs across America: the 2015 bugs without borders survey. Pest World 7: 5–14.

Potter, M. F., J. Fredericks, and M. Henriksen. 2013. Bed bug update persistent pest. Pest Management Professional 81(6): B2–B4.

Pritchard, J. M., and S. W. Hwang. 2009. Severe anemia from bed bugs. Can Med Assoc J 181(5): 287–288.

Puttaraju, H. P., and B. M. Prakash. 2005. Effects of *Wolbachia*-targeted tetracycline on a host-parasitoid-symbiont interaction. Eur J Entomol 102 (4), 669–674.

Rao, H. V., and N. T. Davis. 1969. Sperm activation and migration in bed bugs. J Insect Physiol 15: 1815–1832.

Rao, R. U., L. J. Atkinson, R. P. Vanderwall, and G. J. Weil. 2005. *Brugia malayi*: effects of gamma radiation on adult worms and their intracellular *Wolbachia* bacteria. Exp Parasitol 109: 87–93.

Rasgon, J. L., and T. W. Scott. 2004. Phylogenetic characterization of *Wolbachia* symbionts infecting *Cimex lectularius* L. and *Oeciacus vicarius* Horvath (Hemiptera: Cimicidae). J Med Entomol 41: 1175–1178.

Ray, H. N., and B. Dasgupta. 1955. Observations on the structure and the nature of NR bodies" found in the testes of bed bug. Parasitology 45: 421–425.

Reinhardt, K., R. Naylor, and M. T. Siva-Jothy. 2003. Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ. P Roy Soc Lond B 270: 2371–2375.

Reinhardt, K., R. A. Naylor, and M. T. Siva-Jothy. 2005. Potential sexual transmission of environmental microbes in a traumatically inseminating insect. Ecol Entomol 30: 607–611.

Reinhardt, K., E. Harney, R. Naylor, S. Gorb, and M. T. Siva-Jothy. 2007a. Female-limited polymorphism in the copulatory organ of a traumatically inseminating insect. Am Nat 170: 931–935.

Reinhardt, K., and M. T. Siva-Jothy. 2007b. Biology of the bed bugs (Cimicidae). Annu Rev Entomol 52: 351–374.

Reinhardt, K., R. A. Naylor, and M. T. Siva-Jothy. 2009. Situation exploitation: higher male mating success when female resistance is reduced by feeding. Evolution 63: 29–39.

Reinhardt, K., R. A. Naylor, and M. T. Siva-Jothy. 2011. Male mating rate is constrained by seminal fluid availability in bed bugs, *Cimex lectularius*. PloS ONE 6(7): 10.1371/journal.pone.0022082

Ribaga, C. 1897. Sopra un organo particulare delle cimici dei letti. Riv Patol Veg 5: 223–226.

Rio, R. V. M., G. M. Attardo, and B. L. Weiss. 2016. Grandeur alliances: symbiont metabolic integration and obligate arthropod hematophagy. Trends Parasitol 32(9): 739–749.

Rivnay, E. 1933. The tropisms effecting copulation in the bed bug. Psyche 40: 115–120.

Robinson, W. H., and C. J. Boase. 2011. Bed bug (Hemiptera: Cimicidae) resurgence: plotting the trajectory, pp. 315–318. *In* W. H. Robinson and A. E. de Carvalho Campos (eds.), Proceedings of the Seventh International Conference on Urban Pests, Ouro Preto, Brazil, 7–10 August 2011. Instituto Biologico, São Paulo, Brazil.

Rolinson, G. N., and R. Sutherland. 1965. The binding of antibiotics to serum proteins. Br J Pharmacol Chemother 25(3): 638–650.

Romero, A., M. F. Potter, and K. E. Haynes. 2010. Circadian rhythm of spontaneous locomotor activity in the bed bug, *Cimex lectularius* L. J Insect Physiol 56(11) 1516–1522.

Romero, A., M. F. Potter, and K. F. Haynes. 2009. Behavioral responses of the bed bug to insecticide residues. J Med Entomol 46: 51–57.

Romero, A., M. F. Potter, D. A. Potter, and K. F. Haynes. 2007. Insecticide resistance in the bed bug: A factor in the pest's sudden resurgence? J Med Entomol 44: 175–178.

Ruijter, J. M., C. Ramakers, W. M. Hoogaars, Y. Karlen, O. Bakker, M. J. van den Hoff, and A. F. Moorman. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37(6): e45. doi: 10.1093/nar/gkp045.

Ruknudin, A., and I. A. Silver. 1987. Oxygen tension and sperm migration in the female bed bug. Adv Exp Med Biol 215: 337–343.

Ryne, C. 2009. Homosexual interactions in bed bugs: alarm pheromones as male recognition signals. Anim Behav 78(6): 1471–1475.

Saenz, V. L., R. G. Santangelo, E. L. Vargo, and C. Schal. 2014. Group living accelerates bed bug (Hemiptera: Cimicidae) development. J Med Entomol 51(1): 293–295.

Sakamoto, J. M., and J. L. Rasgon. 2006a. Endosymbiotic bacteria of bed bugs: evolution, ecology, and genetics. Am Entomol 52: 119–122.

Sakamoto, J. M., and J. L. Rasgon. 2006b. Geographic distribution of *Wolbachia* infections in *Cimex lectularius* (Heteroptera: Cimicidae). J Med Entomol 43: 696–700.

Sakamoto, J. M., J. Feinstein, and J. L. Rasgon. 2006. *Wolbachia* infections in the Cimicidae: museum specimens as an untapped resource for endosymbiont surveys. Appl Environ Microbiol 72(5): 3161–3167.

Salazar, R., R. Castillo-Neyra, A. W. Tustin, K. Borrini-Mayorí, C. Naquirá, and M. Z. Levy. 2015. Bed bugs (*Cimex lectularius*) as vectors of *Trypanosoma cruzi*. Am J Trop Med Hygiene 92(2): 331–335.

Sasaki-Fukatsu, K., R. Koga, N. Nikoh, K. Yoshizawa, S. Kasai, M. Mihara, M. Kobayashi, T. Tomita, and T. Fukatsu. 2006. Symbiotic bacteria associated with stomach discs of human lice. Appl Environ Microb 72: 7349–7352.

Scarborough, C. L., J. Ferrari, and H. C. J. Godfray. 2005. Aphid protected from pathogen by endosymbiont. Science 310(5755): 1781–1781.

Shaarawi, F. A. I., W. A. M. Radwan, and S. A. Soliman. 1981. Effects of juvenoids on some biological aspects of the bed bug, *Cimex lectularius* L. I. Effects on female fecundity. Bull Fac Earth Sci (King Abdulaziz Univ.) 5: 123–132.

Shaarawi, F. A. I., W. A. M. Radwan, and S. A. Soliman. 1982. Effects of juvenoids on some biological aspects of the bed bug, *Cimex lectularius* L. II. Effect on egg viability and embryogenesis. Bull Fac Earth Sci (King Abdulaziz Univ.) 6: 35–47.

Siddiqui, S.S., and I. A. Raja. 2015. Molecular detection of endosymbiont bacteria *Wolbachia* in bedbug species *Cimex lectularius* from Vidarbha region of India. Int J Life Sci 3: 200–204.

Siljander, E., R. Gries, G. Khaskin, and G. Gries. 2008. Identification of the airborne aggregation pheremone of the common bed bug, *Cimex lectularius*. J Chem Ecol 34: 708–718.

Silverman A. L., L. H. Qu. J. Blow, I. M. Zitron, S. C. Gordon, and E. D. Walker. 2001. Assessment of hepatitis B virus DNA and hepatitis C virus RNA in the common bedbug (*Cimex lectularius* L.) and kissing bug (*Rodnius prolixus*). Am J Gastroenterol 96: 2194–98.

Siva-Jothy, M. T. 2006. Trauma, disease and collateral damage: conflict in cimicids. Philos T Roy Soc B 361: 269–275.

Stutt, A. D., and M. T. Siva-Jothy. 2001. Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. P Natl Acad Sci USA 98: 5683–5687.

Takahashi, M., and T. Ohtaki. 1975. Ovicidal effects of two juvenile hormone analogs, methoprene and hydroprene, on the human body louse and the bed bug. Jpn J Sanit Zool 26: 237–239.

Tatarnic, N. J., G. Cassis, and M. T. Siva-Jothy. 2014. Traumatic insemination in terrestrial arthropods. Annu Rev Entomol 59: 245–261.

Todd, R. G. 2006. Efficacy of bed bug control products in lab bioassays: Do they make it past the starting gate? Am Entomol 52: 113–116.

Trivedi, M. K., S. Patil, H. Shettigar, K. Bairwa, and S. Jana. 2015. Spectroscopic characterization of chloramphenicol and tetracycline: an impact of biofield treatment. Pharm Anal Acta 6: 395. doi:10.4172/2153-2435.1000395

Tsuchida, T., R. Koga, and T. Fukatsu. 2004. Host plant specialization governed by facultative symbiont. Science. 303(5666): 1989.

Tsuchida, T., R. Koga, M. Horikawa, T. Tsunoda, T. Maoka, S. Matsumoto, J. C. Simon, and T. Fukatsu. 2010. Symbiotic bacterium modifies aphid body color. Science 330(6007): 1102-1104.

Unckless, R. L., L. M. Boelio, J. K. Herren, and J. Jaenike. 2009. *Wolbachia* as populations within individual insects: causes and consequences of density variation in natural populations. Proc R Soc B 276: 2805–2811.

Usinger, R. L. 1966. Monograph of Cimicidae (Hemiptera – Heteroptera). Thomas Say Foundation, College Park, MD.

Van Opijnen, T., and J. Breeuwer. 1999. High temperatures eliminate *Wolbachia*, a cytoplasmic incompatibility inducing endosymbiont, from the two-spotted spider mite. Exp Appl Acarol 23: 871. doi:10.1023/A:1006363604916

Werren, J. H. 1997. Biology of *Wolbachia*. Annu Rev Entomol 42: 587–609.

Werren, J. H., L. Baldo, and M. E. Clark. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6(10): 741–751.

Wilkinson, T. L. 1998. The elimination of intracellular microorganisms from insects: an analysis of antibiotic-treatment in the pea aphid (*Acyrthosiphon pisum*). Comp Biochem Phys A 119: 871–881.

Wittner, M., and R. M. Rosenbaum. 1972. Inhibition and Reversal of Intranuclear RNA Synthesis by Hyperatmospheric Oxygen. J Appl Physiol 33: 820.

Wu, D., S. C. Daugherty, S. E. Van Aken, G. H. Pai, K. L Watkins KL, et al. 2006. Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. PLoS Biol 4(6): e188. DOI: 10.1371/journal.pbio.0040188

Xi, Z., J. L. Dean, C. Khoo, and S. L. Dobson. 2005. Generation of a novel *Wolbachia* infection in *Aedes albopictus* (Asian tiger mosquito) via embryonic microinjection. Insect Biochem Molec 35: 903–910.

Zeh, J. A., M. M. Bonilla, A. J. Adrian, S. Mesfin, and D. W. Zeh. 2012. From father to son: transgenerational effect of tetracycline on sperm viability. Sci Reports, 2, 375. http: //doi.org/10.1038/srep00375

Zhu, F., J. Wigginton, A. Romero, A. Moore, K. Ferguson, R. Palli, M. F. Potter, K. F. Haynes, and S. R. Palli. 2010. Widespread distribution of knockdown resistance mutations in the bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), populations in the United States. Arch Insect Biochem Physiol 73: 245–257.

Zientz, E., T. Dandekar, and R. Gross. 2004. Metabolic interdependence of obligate intracellular bacteria and their insect hosts. Microbiol Mol Biol Rev 68: 745–70.

Zorrilla-Vaca, A., M. M. Silva-Medina, and K. Escandon-Vargas. 2015. Bed bugs, *Cimex* spp.: their current world resurgence and healthcare impact. Asian Pac J Trop Dis 5(5): 342–352.

Zouache, K., D. Voronin, V. Tran-Van, and P. Mavingui. 2009. Composition of bacterial communities associated with natural and laboratory populations of *Asobara tabida* infected with *Wolbachia*. Appl Environ Microbiol 75(11): 3755–3764.

VITA

EDUCATION

B.S. Major: Integrated Biology. May 2008. Brigham Young University, Provo, Utah.

PROFESSIONAL POSITIONS

Technical Director: June 2014 to current. Varment Guard Environmental Services, Inc. Columbus, Ohio.

SCHOLASTIC AND PROFESSIONAL HONORS

National Pest Management Association Intern (2013). Chosen to participate in the National Pest Management Association Annual Meeting Pest World 2013. Phoenix, AZ.

Monsanto Student Travel Award (2012). Money awarded by Monsanto to students and young professionals hosting a symposium at the 60th annual Entomological Association of America annual meeting.

Pi Chi Omega scholarship (2011 and 2010).

3rd prize in student competition at the North Central Branch annual meeting, Entomological Society of America, Minneapolis, Minnesota. (2011).

National Science Foundation Graduate Research Fellowship (2010–2012).

President's Prize in Entomology (2009). First place in the student competition at the 57th Annual National Meeting of the Entomological Society of America, Indianapolis, Indiana.

Graduate School Academic Year Fellowship (2009 and 2008), University of Kentucky Graduate School.

Daniel B. Reedy Quality Achievement Award (2008), University of Kentucky.

William Hebard Scholarship (2007), Brigham Young University College of Agriculture.

Gary M. and Sharra L. Booth Entomology Scholarship (2005), Brigham Young University.

National Merit Scholarship (2004–2007), Brigham Young University.

PEER REVIEWED RESEARCH PUBLICATIONS

Gordon, J.R., **Goodman, M.H**., Potter, M.F. and Haynes, K.F. (2014). Population variation in and selection for insecticide resistance in the bed bug. Sci Reports.4: 3836; DOI: 10.1038/srep03836.

Goodman, M. H., Potter, M. F., and Haynes, K. F. (2012). Effects of juvenile hormone analog formulations on development and reproduction in the bed bug *Cimex lectularius* (Hemiptera: Cimicidae). Pest Manag Sci. doi: 10.1002/ps.3376.

Haynes, K. F., **Goodman, M. H.**, and Potter, M. F. (2010). Bed bug deterrence. BMC Biology 8:117.

Goodman, M. H., and Clark, S. M. (2009). Geographic variability in *Calligrapha verrucosa* (Suffrian 1858), a willow-feeding leaf beetle from western North America (Coleoptera: Chrysomelidae). Insecta Mundi 92: 1–11.

Clark, S. M., Olsen, A. B., and **Goodman, M. H.** (2008). The Subfamily Donaciinae in Utah (Insecta: Coleoptera: Chrysomelidae). Monographs of the Western North American Naturalist 4:1–37.

TRADE JOURNAL ARTICLES

Goodman, M. H. (2016). Aging Bed Bug Populations: How Long Have They Been Here? Pest Control Technol 44:82.

Gordon, J.R., **Goodman, M.H.,** Haynes, K.F. and Potter, M.F. (2014). Trouble brewing for bed

bug insecticides? Pest Control Technol June 2014: 72-80.

Potter, M.F., Gordon, J.R., **Goodman, M.** & Hardin, T. (2013). Mapping bed bug mobility. Pest

Control Technol 41:72-74, 76, 78, 80.

Goodman, M. H., M. F. Potter & K. F. Haynes. (2012). Shedding Light on IGR's and Bed Bugs. Pest Control Technol 40:38, 40-42, 44-46.

Potter, M. F., Haynes, K. F., **Goodman, M. H**., Stamper, S., and Sams, S. (2010). Bed bugs blast from the past. Pest Management Professional 46-52.

PROFESSIONAL PRESENTATIONS

Goodman, M. H. (2015). Endosymbionts in the bed bug, Cimex lectularius. Exit seminar presented to the Department of Entomology at the University of Kentucky, Lexington, KY.

Goodman, M. H., Potter, M. F., and Haynes, K. F. (2013). Bed bug endosymbionts: should they stay or could they go now? National Meeting of the Entomological Society of America, Austin, TX.

Goodman, M. H., Potter, M. F., and Haynes, K. F. (2013). Bed bugs; how long has the infestation been there? University of Kentucky Pest Control Short Course, Lexington, KY.

Goodman, M. H., Potter, M. F., and Haynes, K. F. (2012). Certified bed bug free: Actual results may vary. National Meeting of the Entomological Society of America, Knoxville, TN.

Goodman, M. H., Potter, M. F., and Haynes, K. F. (2012). Bed bug symbionts; traumatic dissemination? National Meeting of the Entomological Society of America, Knoxville, TN.

Goodman, M. H., Potter, M. F., and Haynes, K. F. (2011). The roles of endosymbionts in bed bugs. National Meeting of the Entomological Society of America, Reno, NV.

Goodman, M. H., Potter, M. F., and Haynes, K. F. (2010). Effects of insect growth regulators on the bed bug. North Central Branch Meeting of the Entomological Society of America, Minneapolis, MN.

Goodman, M. H., Potter, M. F., and Haynes, K. F. (2010). Resolving the roles of symbionts in the bed bug. National Meeting of the Entomological Society of America, San Diego, CA.

Goodman, M. H., Potter, M. F., and Haynes, K. F. (2010). Effects of insect growth regulators on the bed bug. University of Kentucky Pest Control Short Course, Lexington, KY.

Goodman, M. H., Andrews, E., Dobson, S., Potter, M. F., and Haynes, K. F. (2009). Characterization and experimental manipulation of endosymbionts in the bed bug. National Meeting of the Entomological Society of America, Indianapolis, IN.

Goodman, M. H., and Booth, G. M. (2008). Sensory studies of a willow-feeding leaf beetle, *Calligrapha verrucosa* (Coleoptera: Chrysomelidae). National Center for Science and Civic Engagement Symposium, Washington, DC.

Goodman, M. H., and Booth, G. M. (2007). Sensory studies of a willow-feeding leaf beetle, *Calligrapha verrucosa* (Coleoptera: Chrysomelidae). National Meeting of the Entomological Society of America, San Diego, CA.

Goodman, M. H., and Clark, S. M. (2006). Geographic variation and sub-speciation in willow-feeding *Calligrapha* (Coleoptera: Chrysomelidae). National Meeting of the Entomological Society of America, Indianapolis, IN.

Goodman, M. H., and Clark, S. M. (2005). Morphological and biological studies of willow-feeding *Calligrapha* beetles, (Coleoptera: Chrysomelidae). National Meeting of the Entomological Society of America, Ft. Lauderdale, FL.