ABSTRACT

Title of Dissertation: UNDERSTANDING THE RELATIONSHIP BETWEEN THE BROWN MARMORATED STINK BUG, *HALYOMORPHA HALYS* (STÅL), AND ITS SYMBIONT, *PANTOEA CARBEKII*, WITH IMPLICATIONS FOR STINK BUG MANAGEMENT

Christopher Michael Taylor, Doctor of Philosophy, 2016

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Symbiotic relationships between insects and beneficial microbes are very common in nature, especially within the Hemiptera. The brown marmorated stink bug, *Halyomorpha halys* Stål, harbors a symbiont, *Pantoea carbekii*, within the fourth region of the midgut in specialized crypts. In this dissertation, I explored this insect-microbe relationship. I determined that the brown marmorated stink bug is heavily reliant on its symbiont, and that experimental removal of the symbiont from the egg mass surface prior to nymphal acquisition led to lower survival, longer development, lower fecundity, and aberrant nymphal behavior. Additionally, I determined that even when the symbiont is acquired and housed in the midgut crypts, it is susceptible to stressors. Stink bugs reared at a higher temperature showed lower survival, longer development, and a cease in egg mass production, and when bugs were screened for

their symbiont, fewer had successfully retained it while under heat stress. Finally, with the knowledge that the stink bug suffers decreases in fitness when its symbiont is missing or stressed, I wanted to determine if targeting the symbiont was a possible management technique for the stink bug. I tested the efficacy of a number of different insecticidal and antimicrobial products to determine whether prevention of symbiont acquisition from the egg mass was possible, and results indicated that transmission of the symbiont from the egg mass to the newly hatched nymph was negatively impacted when certain products were applied (namely surfactants or products containing surfactants). Additionally, direct effects on hatch rate and survival were reported for certain products, namely the insect growth regulator azadirachtin, which suggests that nymphs can pick up residues from the egg mass surface while probing for the symbiont. I conclude that *P. carbekii* plays a critically important role in the survival of its host, the brown marmorated stink bug, and its presence on the egg mass surface before nymphal hatch makes it targetable as a potential management technique.

UNDERSTANDING THE RELATIONSHIP BETWEEN THE BROWN MARMORATED STINK BUG, *HALYOMORPHA HALYS* (STÅL), AND ITS SYMBIONT, *PANTOEA CARBEKII*, WITH IMPLICATIONS FOR STINK BUG MANAGEMENT

by

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Dissertation submitted to the Faculty of the Graduate School of the
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Foreword

With the recommendation of the dissertation director (Charles Mitter) and dissertation committee, as well as the endorsement of the Department of Entomology Graduate Director (Jeffrey W. Shultz), chapter two of this dissertation is included as a previously published work. The citation for this publication is as follows:

Taylor, C. M., Coffey, P. L., Delay, B. D., & Dively, G. P. (2014). The importance of gut symbionts in the development of the brown marmorated stink bug, *Halyomorpha halys* (Stål). *PLoS ONE*, 9(3).

As directed in the graduate catalog for a chapter that was previously published as a coauthored work, I state that I was responsible for the inception of the manuscript and the majority of the manuscript preparation. This work was reformatted to meet the requirements for dissertations, and minor typos and errors were corrected after publication, but all other aspects of the published manuscript, including the use of the first person plural, were used in this dissertation. This publication is cited throughout the dissertation, where appropriate. A letter was sent to the Dean of the Graduate School certifying that inclusion of this previously published work in this dissertation has the approval of the dissertation committee, the dissertation adviser, and the Graduate Director. A copy of this letter can be found in Appendix A.

Dedication

I dedicate this work to the family I am related to by blood that calls me Chris; to the family I am related to by shared experiences and comradery that calls me Bug; and to the aggravating person I have grown to care deeply for that I call Snickerdoodle.

The support that I have received ever since I was young (and more interested in pulling up rocks than playing t-ball) allowed my passion to develop into something that made the completion of my Ph.D. possible. A special thanks to my sister for always being my bug catching assistant when we were younger (and still even now); to my dad for taking me on hikes and to nature centers, for getting special permission for me to black light in parks, and for spending more money than one normally would on water bottles in theme parks and at garden centers when I needed something in which to put a bug that I found; and to my mom, who has been my number one fan since day one, has bought enough Etsy computer bug art for me to start my own gallery, and to this day carries empty coffee cans and bottles in her car in case a cool bug crosses her path.

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A special thank you to both Peter Coffey and Veronica Johnson, both of whom helped me with the tedious and mind numbing tasks of daily stink bug babysitting and DNA extraction for some of the more laborious studies. Their dedication and hard work were major contributing factors to the successful completion of this dissertation.

I'd like to thank my funding sources, without which my time as a graduate student would have been short lived. Thank you to the University of Maryland Department of Entomology for awarding me a Gahan Fellowship all five years of grad school to assist with funding, and thank you to Charles Mitter and Galen Dively for assisting in securing the remainder of my funding through Research Assistantships as well as Teaching Assistantships. This work was also largely supported by a grant from the United States Department of Agriculture-National

Institute of Food and Agriculture (USDA-NIFA) Specialty Crop Research Initiative (SCRI) #2011-51181-30937: Biology, Ecology, and Management of Brown Marmorated Stink Bug in orchard Crops, Small Fruit, Grapes, Vegetables and Ornamentals.

I would also like to specifically thank the coauthors of my chapter two publication, Peter Coffey, Bridget DeLay, and Galen Dively, for their input and assistance with publishing. Bridget developed the primers that made symbiont detection possible in my early studies, and developed the PCR protocols that I used for the duration of my dissertation. Funding was provided through the aforementioned USDA-NIFA grant.

I would finally like to thank my departmental colleagues/ friends past and present, for their support and friendship during the work day as well as after the work day was over. The time spent hanging out, going on collecting trips, having bad bug movie nights, and just venting to each other about our lives was a necessary part of having a healthy grad school experience. In no particular order, I thank: Peter Coffey, Veronica Johnson, Crystal McEwen, Ryan Gott, Gussie MacCracken, Alan Leslie, Becca Wilson, Ashley Jones, Nathalie Steinhauer, Dave Jennings, Erik Bergmann, Andrew Garavito, Lauren Hunt, Brian Lovett, Nancy Harding, Chris Sargent, Dilip Venugopal, and Holly Martinson.

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Chapter 1: Literature review and summary of dissertation findings

Literature Review

Insect: microbe symbioses are common in nature, and many insect orders contain at least some members that rely on a symbiotic microorganism for successful development. Often these symbionts are essential for maintaining the fitness of the host insect, especially when the insect has a very specialized diet, as seen in the blood, sap, or cellulose feeders (Buchner 1965). Many insect microbe interactions were described before the most recent advances in genetic identification techniques. Within the Diptera, all tsetse flies (Glossinidae) harbor symbionts (Buchner 1965) that aid in metabolizing blood meals and promote female fertility (Nogge 1981). Experimental removal of the symbionts leads to stunted growth and lower egg production, as well as sterility in the subsequent generation (Hill and Campbell 1973; Nogge 1976). The termites (Blattodea: Termitoidea) as well as the primitive cockroaches (Blattodea: Cryptocercidae) are unique in their reliance on protozoan gut flora that aid in the digestion of their very high cellulose diet of wood (Cleveland 1925; Andrew 1930; Cleveland et al. 1934; Nalepa 1984). For the members of the order Hemiptera that consume phloem sap (or sometimes uncommonly xylem sap) as their primary food source, one of the adaptations necessary for survival on this specialized diet is the reliance on gut symbionts (Buchner 1965; Douglas 1989; Douglas 2006). Because of the low nitrogen quality of phloem sap, organisms that rely on it must find another way to obtain the necessary amino acids that they themselves cannot produce. The gut symbionts of hemipterans are essential for

maintaining the fitness of these insects because they provide the amino acids and vitamins necessary for development that are lacking in phloem sap (Moran and Telang 1998; Baumann 2005; Douglas 2006; Wu et al. 2006). With recent improvements in genetic identification techniques, more and more of these relationships are being recognized and characterized (Moran et al. 2008), and the number of studies has skyrocketed.

There is substantial diversity regarding symbiont biology, compartmentalization within the host insect, and mode of transmission to future generations within Hemiptera. Firstly, the location of these symbionts within the host varies. Hemiptera: Sternorrhyncha and Auchenorrhyncha store the symbionts within specialized cells or organs (Buchner 1965; Moran and Telang 1998; Moran et al. 2008), while they are found within certain regions of the gut or gastric caeca (also known as crypts) at the posterior end of the midgut in the Hemiptera: Heteroptera (Buchner 1965; Douglas 1989; Otero-Bravo and Sabree 2015). Secondly, almost all phloem feeding hemipterans transmit their primary symbionts vertically (Buchner 1965; Douglas 1989); however, this can be accomplished in a variety of ways: transovarially, orally via infection of materials associated with the egg mass, or via direct smearing onto the egg mass by the mother (Buchner 1965). Because most of the symbiont microbes cannot be successfully cultured on growth media, it is believed that they cannot proliferate outside the host organism due to the highly specialized relationship they share (Bauman and Moran 1997) after coevolving for sometimes hundreds of millions of years with a host (Hosokowa et al. 2006; Takiya et al. 2006; Jousselin et al. 2009). Evolution from free living bacteria to obligate

symbionts has been described as the start of these associations, and this phenomenon is currently being studied in the stinkbug *Plautia stali* (Hosokowa et al. 2016) and the Oriental chinch bug (Itoh et al. 2014), suggesting that some heteropterans are in the process of moving towards a close knit relationship akin to groups such as aphids.

An exception to this rule of vertical transmission has been elucidated by Kikuchi et al. (2007) in the species *Riptortus clavatus* (Hemiptera: Heteroptera: Alydidae) which stores beneficial bacterial symbionts from the genus *Burkholderia* in the gastric caeca. Even though 95-100% of the natural populations of *R. clavatus* studied tested positively for the symbiont (Kikuchi et al. 2005), the egg masses and newly hatched nymphs reared out did not. The nymphs of *R. clavatus* must obtain the fitness improving symbiont from the soil in their habitat every generation (Kikuchi et al. 2007). Kikuchi et al. (2011) surveyed 124 species representing 24 families in the Heteroptera for a similar symbiotic relationship, of which only 39 species in 6 families (all within the superfamilies Lygaeoidea and Coreoidea) tested positively for the environmentally obtained symbiont and showed absence of vertical transmission to the eggs and young. This uncommon method of environmental inoculation by a primary symbiont is only recorded for hemipterans with symbionts in the genus *Burkholderia* (which, unlike vertically transmitted symbionts, were successfully reared in cultures). *H. halys* was among the species that tested negatively for its presence. Therefore it is unlikely that environmental acquisition of a primary symbiont occurs in *H. halys*, since pentatomid stink bug symbionts belong to a distinctly different class of microorganisms (gammaproteobacterial symbionts) than *Burkholderia* (betaproteobacterial symbionts). The patterns support the notion that

most taxonomic groups of heteropterans maintain host- symbiont specificity and compatibility via vertical transmission (Kikuchi et al. 2011).

There is usually a primary (obligatory) symbiont associated with each organism or group of organisms, as well as the possible presence of other secondary facultative symbionts. In the Auchenorrhyncha, there can be numerous primary and secondary symbionts associated with a single species. It should be noted that unlike the primary symbiont(s), the secondary symbionts are shown to have not maintained a close persistent association within the host's lineage like the primary symbionts have (Takiya et al. 2006) and are usually not required by the host to maintain its fitness (Buchner 1965; Moran and Telang 1998; Baumann 2005).

Studies have looked at economically important groups in the Sternorrhyncha such as whiteflies (Aleyrodidae: Zchori-Fein and Brown 2002; Thao and Baumann 2004; Skaljac et al. 2010; Himler et al. 2011; Bing et al. 2012), psyllids (Fukatsu and Nikoh 1998), and mealybugs (Munson et al. 1992; von Dohlen et al. 2001). However within the Sternorrhyncha, the aphids (Aphidoidea) are among the most well studied hemipterans for insect: symbiont interactions. According to Douglas (1998 and 2006), a substantial amount of data has been collected that demonstrates these symbionts provide their aphid hosts with the amino acids that the insects themselves cannot produce from their qualitatively nitrogen deficient diet. Aphids rely on bacteria belonging to the genus *Buchnera* which are strictly vertically transmitted via specialized 'storage cells' known as bacteriocytes (Moran et al. 1993; Wille and Hartman 2009). They are intimately associated with their aphid hosts, and molecular analysis has suggested long-term co-speciation (Moran et al. 1993). Although

Buchnera makes up more than 90% of the bacterial flora found in the bacteriocytes (Haynes et al. 2003), secondary symbionts in genera other than *Buchnera* that are believed to be transmitted vertically and/or horizontally can also occur within aphids (Russell et al. 2003). Recent studies have shown that some of these facultative symbionts actually assist their aphid host in demonstrating resistance to parasitoid wasps (Oliver et al. 2003; Donald et al. 2016), and can protect the aphid while shielding the primary *Buchnera* symbiont from heat stress (Montllor et al. 2002; Burke et al. 2009), demonstrating how complex the symbiont complex may become for some species.

Like the Sternorrhyncha, members of the Auchenorrhyncha pass on their bacteriocyte contained symbionts transovarially (Buchner 1965, Moran and Telang 1998, Moran et al. 2008), and a number of species have been studied, especially within the family Cicadellidae (the leafhoppers). Leafhoppers often exhibit complicated host- symbiont mutualisms, and are often shown to rely on multiple primary as well as secondary symbionts (Takiya et al. 2006). Some aid in such processes as uric acid metabolism (Sasaki et al. 1996), or in the case of the glassy winged sharpshooter *Homalodisca vitripennis*, it is reliant on two species of symbiont, *Baumannia cicadellinicola* which biosynthesizes necessary vitamins, and *Sulcia muelleri* which produces amino acids (Wu et al. 2006). Not only do the symbionts provide their host with their nutritional requirements, but it has also been suggested that the symbionts of leafhoppers and planthoppers may mediate host adaptation to plant resistance (Ferrater et al. 2013).

There are also many heteropterans that share symbiotic relationships with microorganisms. They are typically found within the infraorders Cimicomorpha (namely the sanguinivorous bed bugs [Cimicidae] and assassin bugs [Reduviidae] belonging to the genus *Triatoma*) and Pentatomomorpha, which includes many crop damaging groups such as the stink bugs (Pentatomidae), shield bugs (Scutelleridae), and plataspids (Plataspidae) (Buchner 1965). The bacteria are housed within the gastric caeca in the Pentatomomorpha (Glasgow 1914; Buchner 1965; Dasch et al. 1984), although the method by which vertical transmission occurs varies by group. For some, such as the family Plataspidae, bacteria- containing capsules are deposited with the egg mass and nymphs probe this capsule to become inoculated (Buchner 1965; Fukatsu and Hosokawa 2002; Hosokawa et al. 2005). Some nymphs will probe the symbiont rich fecal material of the adults to become inoculated, as seen in the family Cydnidae (Schorr 1957). For the Pentatomidae and related families, nymphs probe the egg mass which is smeared with the symbiont by the mother (Buchner 1965; Abe et al. 1995; Prado et al. 2006; Kikuchi et al. 2009; Prado and Almeida 2009a, Kaiwa et al. 2010, Bansal et al. 2014, Taylor et al. 2014). This 'post hatch' inoculation behavior that is seen in the above groups is believed to be due to the extracellular nature of the symbiont housing within the gut (Kikuchi et al. 2007), which contrasts the insects which contain their symbionts intracellularly in bacteriocytes and pass them on transovarially. The nymphs stay closely associated with the egg mass, probing the shells or materials for the symbiont. This allows it to colonize the sterile caeca of the midgut within the newly hatched nymphs (Fukatsu and Hosokawa 2002).

Since its accidental introduction approximately 20 years ago (Hoebeck and Carter 2003) the brown marmorated stink bug (BMSB), *Halyomorpha halys* (*Stål*), has become both a household nuisance and agricultural pest in the United States that has steadily been expanding its range (Haye et al. 2015). It is a voracious polyphagous insect that feeds on many vegetable, field and fruit crops, as well as ornamentals (Leskey et al. 2012a, 2012b; Bergmann et al. 2015, 2016). The overwintering habits of *H. halys* also place these insects in close contact with people when they invade homes and other buildings in large numbers (Leskey et al. 2012a). Its rise to pest status brought about an urgent need to study its behavioral and ecological biology in order to develop effective IPM strategies (Leskey et al. 2012a). Nielsen et al. (2008) developed life tables for *H. halys* that showed developmental time and survival differences based on temperature which influenced the decisions regarding rearing conditions for colony management of *H. halys.* Interestingly, Nielsen et al. (2008) saw a difference in overall survival and development when nymphs were allowed to aggregate versus were removed from their egg mass upon hatch at each of the temperatures used.

This dissertation investigated the relationship between the obligate gut symbiont *Pantoea carbekii* and *H. halys*. A species of *Pantoea* was identified from the fourth region of the midgut of *H. halys* by Bridget DeLay and Bill Lamp during 2013 studies looking into the possible presence of a symbiont. They were found in the region of the gut that is the typical location of obligate symbionts in other stink bug species (Buchner 1965). Bansal et al. (2014) then published on this symbiont and proposed the species name *Candidatus Pantoea carbekii* as a distinct species within

the genus *Pantoea*, and confirmed its presence in *H. halys* populations across the United States. Kenyon et al. (2015) then published on the symbionts genome and visualized the bacteria's presence on the egg mass surface. Their work on the *P. carbekii* genome showed similarities between *P. carbekii* and other obligate symbionts. *P. carbekii* exhibited a reduced genome when compared to similar free living bacterial species, and amino acid biosynthesis genes as well as stress response genes were present that were similar to those seen in other obligate symbionts (Kenyon et al. 2015). Research into such a potentially important association between symbiont and host for *H. halys* was lacking though and therefore necessary to obtain a more thorough understanding of *H. halys* biology.

Summary of Dissertation Findings

The second chapter of my dissertation examined the effects on *H. halys* fitness when symbiont acquisition was prevented via chemical sterilization of the egg mass. It is possible to surface sterilize the egg masses of pentatomids, preventing the nymphs from acquiring the gut symbionts (Fukatsu and Hosokawa 2002; Prado et al. 2006; Prado and Almeida 2009a), and a number of studies demonstrate that the experimental removal of the symbiont can lead to negative impacts on the host, such as a decrease in fitness, retarded development and/or higher nymph mortality (e.g. Plataspidae: Fukatsu and Hosokawa 2002; Pentatomidae: Abe et al. 1995; Prado and Almeida 2009a; Parastrachiidae: Kashima et al. 2006; Cydnidae: Schorr 1957; Acanthosomatidae: Kikuchi et al. 2009). However these patterns of negative impacts on the host are not universally true and it appears that there are varying degrees of

mutualisms between the symbiont and its host, at least within the Pentatomidae. In some cases surface sterilization experiments showed no decrease in fitness, as seen in the southern green stink bug *Nezaria viridula* (Prado et al. 2006) and the harlequin bug *Murgantia histrionica* (Prado and Almeida 2009a). For *Chinavia hilaris* (formerly *Acrosternum hilare*) however, the data suggests that the symbiont is required for adequate development (Prado and Almeida 2009a).

My research demonstrated that there were significant impacts on survival, development, and fecundity of *H. halys* when deprived of its symbiont (Taylor et al. 2014). *H. halys* nymphs reared from sterilized egg masses took significantly longer to develop, had significantly lower survival, and significantly lower fecundity (i.e. they had a longer pre-oviposition period and laid fewer eggs than those in the control treatment). The egg masses laid by adults that came from the chemically sterilized eggs also had a significantly lower clutch size and hatch rate when compared to the egg masses laid by control adults. Only one bug made it to the adult stage from all the egg masses laid by sterile treatment *H. halys*, indicating negative effects on progeny of aposymbiotic *H. halys* in the subsequent generation. There was also an effect on first instar behavior. Sterile treatment bugs stayed clustered around their egg mass significantly longer than control bugs did. My results showed that *H. halys* is indeed dependent on its obligate gut microbe.

With the knowledge that the symbiont of *H. halys* is important for its survival, I next wanted to determine whether it could be influencing where *H. halys* could thrive. Venugopal et al. (2016) determined that at broad spatial scales, temperature was the factor that drove *H. halys* populations (i.e. the hotter it is, the fewer stink

bugs were present), but whether this is due to constraints put solely on *H. halys* or its symbiont as well was unknown. Wernegreen (2012) suggested that, due to the genome reduction of many obligate symbionts, they are vulnerable to heat stress and this can put constraints on the host insect that might not necessarily be as prone to heat stress itself. Symbiont loss at higher temperatures was demonstrated in *Nezara viridula* (Prado et al. 2009), *Chinavia hilaris* and *Murgantia histrionica* (Prado et al. 2010), indicating a clear sensitivity to heat changes by the symbiont of other stink bug species which has the potential to mediate host fitness when the respective host depends on its symbiont.

My third chapter looked at the different impacts of temperature stress on the symbiont, first while it is present on the egg mass surface, and then once it is present in the gut of *H. halys*. When egg masses on green bean plants were exposed to one of three different summer day conditions (mimicking temperature and humidity fluctuations on a normal, above average, or extreme summer day for Maryland), the egg masses reared at the extreme conditions had significantly lower hatch rate than the normal and above average conditions, which demonstrated a direct effect on egg survival. There were no differences in hatch rate between the normal and above average condition egg masses. Once hatched, the nymphs were reared to adulthood at a constant 25° C, and numerically lower survival to adulthood was seen in the above average versus normal condition egg mass treatments (despite only being exposed to the different conditions at the egg stage and showing no initial differences). However the adults screened tested positively for the symbiont across all three treatments, indicating that the above average and even extreme conditions were not able to

completely kill the symbiont located on the egg mass surface. A follow up experiment exposed egg masses to the same normal and above average conditions, but the second instars were screened for the symbiont to determine if the ratio of inoculated to not inoculated was different by treatment. Results showed that the nymphs were just as successful at obtaining their symbiont under both conditions, indicating that the bacteria are able to survive equally well under both normal and above average summer conditions in Maryland. My studies did not show heat stress impacted the symbiont in such a way that acquisition was disrupted by newly hatched nymphs, at least under conditions that do not directly impact *H. halys* itself. The egg laying behavior of females, who lay their eggs on the underside of leaves, may benefit the bacteria by providing a cool microclimate that allows them to survive on the egg mass until nymphal hatch and inoculation.

I also conducted a study where I looked at the effect of heat stress on bugs from control versus sterilized egg masses. Egg masses were either left alone or chemically sterilized, all reared at 25° C until hatch, and then post hatch reared at either 25 or 30° C to determine whether fitness and symbiont survival decreased at higher temperatures once the bug has been colonized by its symbiont. Bugs from sterilized egg masses had lower survival, longer development and lower fecundity than their control counterparts at both temperatures, results similar to what was seen in chapter two. Interestingly, control bug survival was lower at 30° C versus control bug survival at 25° C, and egg mass production all but ceased when control bugs were reared at the higher temperature. PCR analysis determined that fewer control bugs retained their symbiont at the higher temperature versus those reared at the

lower temperature, results similar to those seen in studies conducted by Prado et al. (2009 and 2010). Taken together, these results support the hypothesis that prolonged heat stress has the capability to impact symbiont survival within the bug, and likely puts constraints on the bug itself.

Because of how dependent *H. halys* is on the presence of *P. carbekii*, the symbiont can be viewed as a weak link in the biology of *H. halys*, and may be an avenue worth exploring as a management technique for *H. halys*. Although targeting or manipulating the symbiont has been suggested as a possible management strategy (Douglas 2007; Prado and Zucchi 2012), real world applications of an egg mass sterilization method are lacking in the literature. Mathews and Barry (2015) did show impacts on hatch rate and early instar survival when *H. halys* egg masses were treated with compost tea, but whether this impacted the symbionts on the egg mass surface was not verified and it may have been due to the nymphs picking up residues from the egg mass surface during their probing behavior.

For my fourth chapter, I decided to look at the possible sterilizing effects of commercially available products that can be applied as a liquid spray. For a laboratory study, I chose six different commercially available products (antimicrobials, insecticides, and surfactants) to determine whether egg mass exposure would show any direct effects on hatch rate and survival and/ or any effects on symbiont acquisition success. In the laboratory study, hatch rate was significantly reduced by two of the six treatments, Agri-Mycin 17 (an antimicrobial with the active ingredient streptomycin sulfate) and Naiad (a mix of ionic and nonionic surfactants). Survival was significantly impacted by two of the six treatments, AzaGuard (an insect

growth regulator with the active ingredient azadirachtin) and Naiad. The success of symbiont acquisition was disrupted by three of the six treatments, Naiad, AzaGuard and Liquid Copper Fungicide (an antimicrobial with the active ingredient copper diammonia diacetate complex). The surfactant Naiad was the most successful product, which impacted all three parameters being measured. Hatch rate was significantly reduced (91.3% in control versus 60% in Naiad treatment), survival from first to second instar was significantly reduced (82% in control to 65.9% in Naiad treatment), and inoculation rate by egg mass was significantly reduced (89% in control versus 23.6% in Naiad). Although successful, sterilization was not complete. The egg mass coating under which the symbiont is located (Kenyon et al. 2015) likely protects the symbiont from exposure, especially since a few of the antimicrobials that should have had an effect didn't impact symbiont survival and transmission at all, but the surfactant (as well as products containing surfactants such as AzaGuard) worked well. It is likely that surfactants can break down the egg mass coating and expose and or kill the symbiont located underneath.

I also conducted a small scale field study in a peach orchard to determine whether similar results were obtainable under field conditions when products were applied with a backpack airblast sprayer, but replication was low due to low *H. halys* populations and colony rearing issues, and I was only able to use a limited treatment number. Small but significant differences in hatch rate were detected for the surfactants Naiad and Triton-X-100 (a laboratory nonionic surfactant), but I saw no significant differences in survival or inoculation. Nevertheless, there is potential for

future research into fine tuning field applications of products to achieve coverage levels necessary to penetrate the egg mass coating and affect the symbiont.

My dissertation has furthered our understanding of insect microbe interactions by studying aspects of the *H. halys*- symbiont relationship. It has also revealed a potential avenue of managing *H. halys* by directly targeting the symbiont and treating it as a weak link in the life history of *H. halys*.

Chapter 2: The importance of gut symbionts in the development of the brown marmorated stink bug

Abstract

The invasive brown marmorated stink bug, *Halyomorpha halys* (Stål), has become a severe agricultural pest and nuisance problem since its introduction in the U.S. Research is being conducted to understand its biology and to find management solutions. Its symbiotic relationship with gut symbionts is one aspect of its biology that is not understood. In the family Pentatomidae, the reliance on gut symbionts for successful development seems to vary depending on the species of stink bug. This research assessed the role of gut symbionts in the development, survivorship and fecundity of *H. halys*. We compared various fitness parameters of nymphs and adults reared from surface sterilized and untreated egg masses during two consecutive generations under laboratory conditions. Results provided direct evidence that *H. halys* is negatively impacted by the prevention of vertical transmission of its gut symbionts, and that this impact is significant in the first generation and manifests dramatically in the subsequent generation. Developmental time and survivorship of treated cohorts in the first generation were significantly affected during third instar development through to the adult stage. Adults from the sterilized treatment group exhibited longer pre-oviposition periods, produced fewer egg masses, had significantly smaller clutch sizes, and the hatch rate and survivorship of those eggs were significantly reduced. Observations following hatch of surface sterilized eggs also revealed significant effects on wandering behavior of the first instars. The second generation progeny from adults of the sterilized cohorts showed significantly lower

survival to adulthood, averaging only 0.3% compared to 20.8% for the control cohorts. Taken together, results demonstrate that H. halys is heavily impacted by deprival of its gut symbionts. Given the economic status of this invasive pest, further investigations may lead to management tactics that disrupt this close symbiotic relationship in the biology of *H. halys*.

Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is a highly polyphagous pest species (Leskey et al. 2012a) indigenous to northeastern Asia where it damages various trees, vegetables, and leguminous crops (Hoebeck and Carter 2003). It was first identified as an invasive pest in the U.S. in 2001 but previous reports of its presence in eastern Pennsylvania date back to 1996 (Hoebeck and Carter 2003). Since its introduction, *H. halys* has rapidly expanded its range and has now been detected in 40 states (State by State 2014). Populations have steadily increased in the northeast U.S., where it has become a severe agricultural pest in six states (Kuhar et al. 2012; Leskey et al. 2012b) and a nuisance problem in 13 states due to its en masse overwintering habits in structures (Hamilton 2009; Leskey et al. 2012a). As an invasive species, there is limited information on *H. halys* and its impacts on U.S. agriculture and the environment in North America. With funding from USDA and commodity organizations, several working groups of more than 60 researchers are conducting a diverse range of studies to understand the biology and ecology of this pest and to find management solutions.

Like many insects in the order Hemiptera that share symbiotic relationships with microorganisms, *H. halys* is presumed to share a close symbiotic relationship with gut flora. Newly hatched nymphs aggregate and stay closely associated with the egg mass for several days, probing the egg chorion for the symbionts which are thought to colonize the sterile caeca of the midgut. The importance of the gut symbionts to the development and survival of *H. halys* is not known. Symbiotic relationships in the Hemiptera are well documented in the Auchenorrhyncha and Sternorrhyncha and are typically found within the diet specific heteropteran infraorders Cimicomorpha (namely the sanguinivorous bed bugs [Cimicidae] and assassin bugs [Reduviidae: *Triatoma*]) and Pentatomomorpha, which includes many agriculturally important families such as the stink bugs (Pentatomidae), shield bugs (Scutelleridae), and plataspids (Plataspidae) (Buchner 1965). The gut symbionts are essential for maintaining the fitness of many hemipterans that have been studied because they provide the amino acids and vitamins necessary for development that are lacking in phloem sap (Baumann and Moran, 1997; Moran and Telang 1998; Douglas 2006). The bacteria are housed within the gastric caeca in the Pentatomomorpha families (Glasgow 1914; Buchner 1965; Dasch et al. 1984), although the method by which vertical transmission occurs varies. For some, such as the family Plataspidae, bacteria-containing capsules are deposited with the egg mass and nymphs probe these capsules to become inoculated (Buchner 1965; Fukatsu and Hosokowa 2002; Hosokowa et al. 2005). Some nymphs will probe the symbiont rich fecal material of the adults to become inoculated, as seen in the family Cydnidae (Schorr 1957). For the Pentatomidae and related families, nymphs probe the egg mass

which is coated with the symbiont by the mother (Buchner 1965; Abe et al. 1995; Prado et al. 2006; Kikuchi et al. 2009; Kaiwa et al. 2010). This 'post hatch' inoculation behavior is believed to be due to the extracellular nature of the symbiont housing within the gut (Kikuchi et al. 2007).

Sterilization has been used in studies to prevent the nymphs from acquiring the deposited gut symbionts in order to study the effects of symbiont deprivation (Fukatsu and Hosokowa 2002; Prado et al. 2006; Prado and Almeida 2009a). Some studies have demonstrated that experimental removal of the symbiont can lead to negative impacts on the hemipteran host, such as a decrease in fitness, slowed development and/or higher nymph mortality (e.g. Plataspidae: (Fukatsu and Hosokowa 2002); Pentatomidae: (Abe et al. 1995; Prado and Almeida 2009a; Kikuchi et al. 2012); Parastrachiidae: (Kashima et al. 2006); Cydnidae: (Schorr 1957); Acanthosomatidae: (Kikuchi et al. 2009). In a very specialized case, the subsocial *Parastrachia japonensis* females care for the eggs and secrete symbiont rich mucus less than an hour before synchronous nymphal hatch. When females are removed, vertical symbiont transmission is prevented and eggs hatch asynchronously (Hosokowa et al. 2012a). However the patterns of negative impacts on the host after removal of the symbiont are not universally true and it appears that there are varying degrees of reliance on the symbiont by its host, at least within the Pentatomidae. Surface sterilization experiments showed no difference in developmental time over two generations in the southern green stink bug *Nezara viridula* (Prado et al. 2006). The harlequin bug *Murgantia histrionica* saw a longer generation time but a slight increase in survivorship when its symbionts were removed (Prado and Almeida

2009a). Environmental factors may also play a role, as high temperature was shown to negatively affect the symbionts and was coupled with a decrease in fitness in *M. histrionica* and the green stink bug *Chinavia hilaris* (formerly *Acrosternum hilare*) (Prado et al. 2010). For *C. hilaris*, the symbiont is required for adequate development (Prado and Almeida 2009a), which suggests that temperature effects on the symbionts can negatively impact the host in these cases.

It is apparent that there is no distinct trend available to gauge the level of dependency on gut symbionts in the Pentatomidae. Although most stink bug symbionts were found to belong to the clade to which the bacterial genera *Erwinia* and *Pantoea* belong, the relationships between them are complicated and symbiont monophyly doesn't occur above the pentatomid genus level (Prado and Almeida 2009b). This complexity is seen in other Heteropteran groups as well, such as the families Cydnidae (Hosokowa et al. 2012b) and Scutelleridae (Kaiwa et al. 2011). Therefore, these symbiotic relationships must be studied on a species to species basis. The purpose of this research was to assess the role of gut symbionts on the development, survivorship and fecundity of *H. halys*. We compared various fitness parameters of nymphs and adults reared from surface sterilized and untreated egg masses under laboratory conditions, and determined the importance of the gut symbionts. An understanding of the role and complexities of symbiotic relationships in the Heteroptera could lead to possible exploitation as a strategy for managing pestiferous species like *H. halys* (Prado and Zucchi 2012).

Materials and Methods

Ethics statement

The wild caught *Halyomorpha halys* used to start the lab colony before the experiment were collected in soybean fields at the University of Maryland Beltsville Research Farm where we have permission to conduct research and collect samples. No specific permission was required to collect *H. halys*, and no endangered or protected species were involved.

Insect culture

A laboratory colony of *H. halys* was established in January 2012 with adults collected from soybean fields at our University of Maryland Beltsville Research Farm during the previous fall and held for several months at 12±0.5° C to break diapause. The insects were reared on potted plants of *Phaseolus vulgaris* L., excised bean pods, and raw sunflower seeds in mesh cages (60×30×35 cm). The colony was maintained for three generations in walk-in environmental chambers at 25° C, RH of 65±5%, and a 16 h L: 8 h D photoperiod. Egg masses laid within 24 hours were collected from bean plants from several cages of adults exhibiting peak oviposition. A 15 mm leaf disc containing each egg mass was cut from the bean leaves using a cork borer.

Egg mass treatments

Thirty-six egg masses, each consisting of 28 eggs representing the median clutch size (Nielsen et al. 2008) were randomly divided into two treatment groups. One group was surface sterilized in 10% Clorox solution to remove the symbionts present. To determine the maximum soaking time for *H. halys* egg masses, we conducted a preliminary experiment involving groups of egg masses soaked for either 2, 4, 6, or 8 minutes in 10% Clorox solution. Results showed that 6 minutes in the

bleach solution was the longest soaking time allowed before *H. halys* eggs began to dissociate from their egg mass, which can negatively impact hatch rate (Lockwood and Story 1998). Using this soaking time and a modified protocol by Prado and Almeida (Prado et al. 2006; Prado and Almeida 2009a), each egg mass was submerged in a 100% ethanol bath for 5 minutes; then submerged in a 10% Clorox bleach solution for 6 minutes; and then rinsed, first in a separate ethanol bath and then in a distilled water bath. The control group of 18 egg masses was not manipulated in any way.

Nymphal rearing (generation 1)

Each egg mass was reared in a clear (16x14x5 cm) plastic deli dish, with a screened opening (8 cm diameter) in the lid to provide ventilation. Each dish contained an excised organic bean pod and a 15 ml floral water pick to provide a transportable water supply for an inserted leaf terminal of *P. vulgaris*. The deli dishes were held in an environmental chamber set at 25° C, 65-75% humidity, and a 16 h L: 8 h D diurnal cycle. To account for possible environmental gradients within the chamber, six cohorts of each treatment were randomly assigned to each of the three shelves which were treated as a blocking factor. The dishes were lightly misted with distilled water every day, and fresh leaf terminals and bean pods were provisioned every 4-6 days. Data were recorded each day on the number of live stink bugs of each developmental instar. Dead insects were also recorded and removed each day. Time expressed in developmental days was recorded when each egg mass hatched (Day 1) to synchronize development across all cohorts.

Insect behavioral observations (generation 1)

Because symbiont presence or absence may influence the behavior of newly-hatched nymphs (Hosokowa et al. 2008), the behavior of the first instars was recorded until molting to the second instar began. The number of nymphs clustered around and physically touching the egg mass compared to the number wandering was recorded for each cohort during days 1-5. Nymphs were not manipulated or prodded to prevent behavior modification during this time.

Adult rearing (generation 1)

Adult stink bugs produced in the replicate cohorts of each treatment were collected and either stored for PCR analysis or transferred to larger rearing cages to monitor survival and oviposition. It was not possible to maintain the replication of individual cohorts, so adults were pooled from the six cohorts of each treatment that were reared on each shelf of the rearing chamber. Because there was a significant blocking effect, nymphs in cohorts reared on the lowest shelf produced insignificant numbers of adults for further testing, although the control cohorts still performed better than the sterilized cohorts. Thus, only four rearing cages were established by pooling adults collected from the six cohorts of each treatment from the upper two shelves. This was necessary to avoid overcrowding in the rearing cages, as well as to track any possible blocking effect that might carry over to the second generation.

Adults were reared under the same environmental conditions in the same mesh cages as described above for the laboratory colony. Records were kept each day on the number of new adults added and the total number of adults per cage. Dead adults were also recorded and removed daily. Similar to colony rearing, cages were provisioned with six potted bean plants, bean pods, and shelled sunflower seeds to

provide an additional protein source. Plants and supplemental foods were replaced every 4-6 days as needed.

Egg mass selection and nymphal rearing (generation 2)

Plants and mesh surfaces of the adult cages were checked daily for egg masses. Because developmental time to adulthood varied among replicate cohorts, new adults were added to the rearing cages over the course of several weeks. Subsequently, egg masses were produced continuously in the rearing cages, once *H. halys* females passed the 2 week pre-oviposition period (Nielsen et al. 2008). Adults from the control group produced considerably more eggs over time than adults from the sterilized group. To provide a representative selection of egg masses for generation 2 rearing, only one egg mass was chosen every other day from each control cage until 12 masses were collected. Ultimately, a total of 24 replicate egg masses from the control group were selected for generation 2 rearing. Both cages of adults reared from sterilized eggs produced only 15 egg masses total and all were chosen for generation 2 rearing. Each egg mass on a 15 mm leaf disc was placed in a deli dish containing a bean pod and leaf terminal of *P. vulgaris*, and maintained following the same protocol used for generation 1 rearing. No manipulation of the egg masses was performed for either treatment. To account for possible environmental gradients in rearing, six deli dishes of each treatment group were placed on each of two shelves in two separate environmental chambers based on the shelf from which the adults that laid them came. Similar to the data recorded in generation 1, daily records for each cohort were kept on the number of live and dead nymphs of each developmental instar. To track and compare development of cohorts since they were set up over several weeks in each treatment group, day 1 in

developmental time commenced when each egg mass hatched like in generation 1. All other egg masses from the control cages that were not used for generation 2 rearing were also collected and hatched in deli dishes to provide additional data on the time to hatch, clutch size, and level of hatchability.

PCR analysis

Prior studies isolated two symbionts from gut dissections of field caught *H. halys* adults and primers were developed for their detection (unpublished data). One symbiont was a species of *Wolbachia*, while the second was *Pantoea agglomerans*. PCR detection of the *Wolbachia* symbiont was unsuccessful on multiple trials, but was successful for *P. agglomerans* (Note that after this study was published, the *Pantoea* symbiont found within *H. halys* was found to be a new, different species than *P. agglomerans*, and was named *P. carbekii* in a publication by Bansal et al. 2014. Within this chapter it is referred to as *P. agglomerans*, but in all other sections of this dissertation it is referred to as *P. carbekii*).

To verify the presence of *Wolbachia* and *P. agglomerans* in adults and egg masses from the colony, samples of both life stages were preserved in 100% ethanol-filled centrifuge tubes and stored for later DNA extraction. To substantiate whether egg masses were successfully sterilized and the sterilization effect carried over to the adult stage, subsamples of male and female adults from treatment groups of both generations, as well as remains of every hatched egg mass from the treatment groups were also stored for DNA extraction. All samples were extracted for DNA using a Quiagen DNEasy Blood and Tissue Kit. The entire egg mass or remains was used for extraction, while only the abdomen and gut contents of adult stink bugs were removed and well macerated in the collection tube prior to DNA extraction to allow

for optimal lysing. Each individual adult was analyzed separately and gel band presence or absence was determined for each. We also used a NanoDrop Spectrophotometer to analyze DNA concentrations of a subset of adult and egg mass husk extractions to determine whether adequate amounts of DNA were extracted for PCR. This was mainly done for the egg mass husks, which may have been devoid of microbes by the time the nymphs had moved off of them and they were stored for analysis.

Data Analysis

PCR data analysis between treatments was limited due to sample size, but a One-Tailed Fishers Exact Test was used to analyze differences between generation 1 adults by treatment. The effect of sterilization on nymph and adult development of *H. halys* in days from egg hatch was tested for each generation using a one-way analysis of variance (Proc Mixed, SAS). For survivorship, a two way ANOVA was used to analyze the treatment, generation and interaction effects on each stage, after an arcsine square-root transformation of the percentage data. In both analyses, each stage was analyzed separately. Several variables of egg mass production (egg masses per female, clutch size, days to hatch, and percent hatchability) were analyzed separately using a one-way ANOVA. The behavior movement of first instar from the egg mass was tested for both treatment and treatment by time effects after adjustments were made for auto-correlation between days. All count data were tested for lack of normality and either log- or square root-transformed prior to statistical analyses. In certain analyses, a random blocking factor was included in the model to remove experimental variance due to the position of the deli dishes within the rearing

chambers. For mean comparisons, Tukey-Kramer's test was used to calculate adjusted P values for differences.

Results

Spectrophotometer and PCR analysis

PCR detection of the *Wolbachia* symbiont was unsuccessful on multiple trials. From this point forward, all reference to the symbiont refers to the second species, *P. agglomerans* unless otherwise stated. Colony adults showed a DNA concentration between 190-240 ng/µl and strong bands were detected on the running gel for *P. agglomerans*. For untreated egg masses collected directly from the colony, one sample showed a 90 ng/µl concentration and a weak gel band, while the other showed a -1.1 ng/µl concentration with no band for *P. agglomerans*. These results suggested that individual egg masses might not be good indicators of symbiont presence or absence. To maximize DNA yield for DNA extraction, we pooled chorions of hatched egg masses by block for both treatment groups of generation 1. However, even with pooling and a lower final elution amount to increase DNA concentration, all samples showed between 0 to 8 ng/µl of DNA. We then used PCR to test for the presence of *P. agglomerans* on unhatched control and sterilized egg masses pooled in groups of two to increase DNA yield. Using lower elution amount during the extraction process, control egg masses showed distinctly bright gel bands for the symbiont, which confirmed its presence on egg masses, whereas the sterilized egg masses showed qualitatively weak gel bands, indicating that either some bacterial cells survived the sterilization treatment, or residual bacterial DNA was not completely rinsed off of the egg masses (and trapped between eggs). Based on these findings, PCR was not

performed on the generation 1 and 2 egg mass chorions because we concluded that they lacked sufficient DNA for accurate PCR detection.

For the adult extractions, 21 of the 24 (87.5%) control adults of the first generation tested positively, while only 7 of 18 (38.9%) tested positively in the sterilized treatment group. This difference in adult inoculation rates between treatment groups was significant ($P < 0.001$, One-Tailed Fisher's Exact Test). For the second generation, since survivorship to adults was very low in the sterilized treatment, we conducted PCR analysis on only one surviving adult but also tested samples of individual second and fifth instar nymphs from the sterilized group. Of these samples, the adult, two of the four fifth instars, and three of the six second instars tested positively for *P. agglomerans*, although the gel bands were qualitatively weak in all positive samples. Eight randomly selected generation 2 adults from the control treatment were selected to verify carryover from generation 1, and all tested positively for *P. agglomerans*.

Developmental time

Since the exact age of egg masses was unknown (all laid within 24 hours) and most neonates hatched in synchrony within a few hours, exact egg development prior to hatch could not be determined. For the subsequent nymphal instars and adults, development time was expressed as the number of days from egg hatch (Day 1) that was required to reach the peak density of individuals of each stage. ANOVA results showed that the surface sterilization treatment resulted in an overall delay in development, except for development to the second instar (Figure 2.1, Table 2.1). Nymphs from sterilized egg masses took significantly longer to develop to the third instar than nymphs from untreated control eggs, and differences in development times

significantly increased with each subsequent life stage. Time to peak numbers of adults from sterilized eggs averaged 62.9 days from egg hatch, 8.1 days later than the control group.

The same analysis was performed on the of development time of generation 2 nymphs hatching from egg masses produced by adults from the control and sterilized groups in generation 1. These eggs were not manipulated in any way. However, few progeny from the sterilized adults survived after the second instar. Only the data on development of the second instars was available for statistical analysis, which showed no difference between cohorts that developed from egg masses laid by adults from the control and sterilized first generation cohorts (Figure 2.1, Table 2.2).

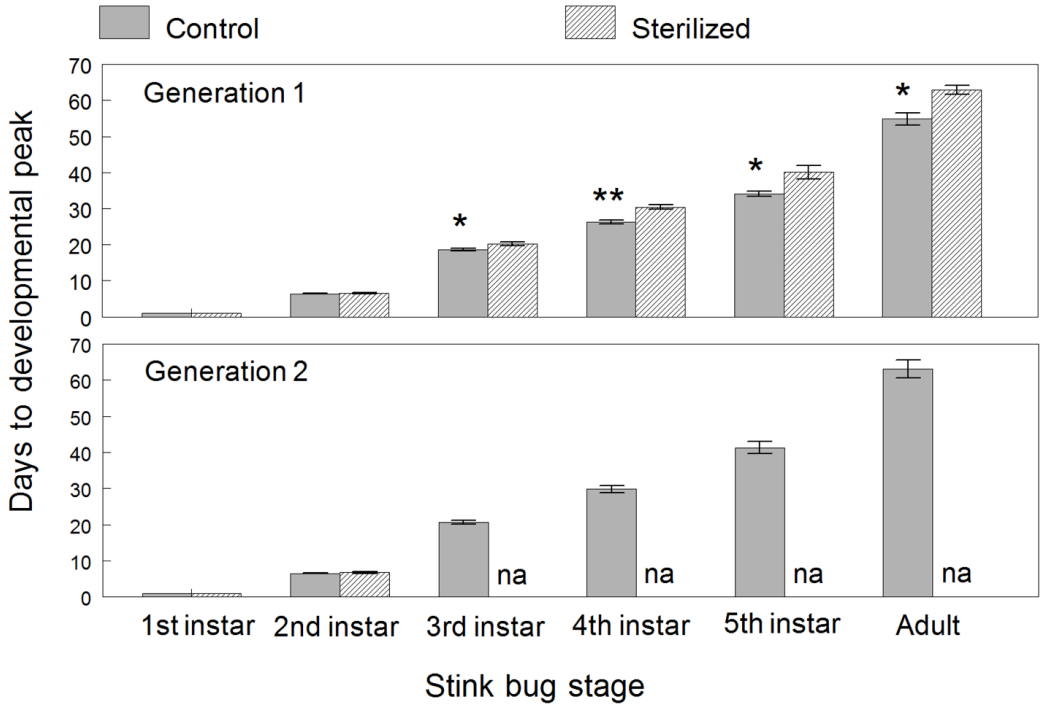

Figure 2.1. Development time expressed as the number of days from egg hatch required to reach peak density of each stage of *H. halys*. Means (± SEM) are plotted for first generation cohorts reared from sterilized and untreated control eggs and for subsequent second generation cohorts. An asterisk over each pair of means for each developmental stage indicates a significant difference ($P < 0.05$); two asterisks indicate a very significant difference ($P < 0.001$). 'NA' indicates that no data were available.

Table 2.1. Developmental time by life stage for generation 1

Developmental stage	***Control group Mean (days)***	***Sterile group Mean (days)***	***Df***	***F***	***P***
1st instar	NA	NA	-	-	-
2nd instar	6.4 ± .12	6.5 ± .12	1, 32	0.46	0.50
3rd instar	18.6 ± .34	20.2 ± .54	1, 31	11.69	0.002
4th instar	26.2 ± .55	30.4 ± .60	1, 29	32.59	<0.001
5th instar	34.1 ± .73	40.1 ± 1.83	1, 29	11.31	0.002
Adult (males)	50.6 ± 1.52	60.5 ± 1.86	1, 23	16.45	<0.001
Adult (females)	51.5 ± 2.17	59.3 ± 1.51	1, 24	6.73	0.016
Adult (total)	54.8 ± 1.65	62.9 ± 1.31	1, 27	11.77	0.002

Table 2.2. Developmental time by life stage for generation 2

Developmental stage	***Control group Mean (days)***	***Sterile group Mean (days)***	***Df***	***F***	***P***
1st instar	NA	NA	-	-	-
2nd instar	6.5 ± .12	6.8 ± .25	1, 31	1. 25	0.27

Survivorship

The percent survival to each nymph instar and adult stage was calculated by dividing the total number of eggs per mass in each cohort by the peak density of individuals that reached each developmental stage. This value was considered a relative estimate of survival since development through the instars was continuous and thus the peak density may be less than the absolute numbers of individuals going through the developmental stage. Nevertheless, the relative estimates given in Table 2.3 and Figure 2.2 provided a means to compare survival between treatments and generations.

Survival to the first instar was indicated by the mean percentage of egg hatch which ranged from 89 to 92% in generation 1 and was not significantly different

between treatments ($t_{(1,71)}$ = .27, PADJ = 0.99). This is evidence that the surface sterilization method had no effect on the hatchability of eggs compared to the control eggs. However, despite the fact that eggs were not sterilized in generation 2, the mean percent hatch of the 15 egg masses laid by adults reared from the sterilized cohorts in generation 1 was significantly lower than the hatchability of eggs from adult control cohorts ($t_{(1,71)}$ = 3.73, PADJ = .002). This was further shown by a significant interaction effect for first instars (Table 2.3).

For the other developmental stages during both generations, cohorts of *H. halys* from sterilized eggs or from untreated eggs laid by adults that were reared from sterilized eggs showed significantly lower survivorship than the cohorts from the control group (Figure 2.2, Table 2.3). The second generation progeny from adults of the sterilized cohorts also showed significantly lower survivorship in all developmental stages compared to the sterile cohorts in generation 1 (2nd instar: $t_{(1,71)}$ = 4.17, PADJ < .001; 3rd instar: $t_{(69)}$ = 7.77, PADJ = <.001; 4th instar: $t_{(70)}$ = 8.01, PADJ = <.001; 5th instar: $t_{(69)}$ = 6.82, PADJ = <.001; adults: $t_{(69)}$ = 6.70, PADJ = <.001). Differences in survivorship between the sterilized and control cohorts depended on the generation but this interaction was not significant for all developmental stages. The treatment by generation interaction was significant for developmental stages after the second instar, except fifth instars, and the general trend showed a more pronounced lower survival of older nymphs reared from eggs of generation 1 adults of the sterilized group. The generational survival is reflected by the number of individuals that survived to the adult stage relative to the total number of eggs. Survival to adults from the control and sterilized egg masses in the first

generation averaged 24.8% and 15.7%, respectively, and the difference was significant ($t_{(68)}$ = 3.02, PADJ = .018). The rate of mortality was much greater during the second generation of progeny reared from adults of the sterilized group. The average generational survival of these cohorts was only 0.3% and significantly lower than the generational survival of 20.8% for the control cohorts ($t_{(69)}$ = 7.71, PADJ = <.0001).

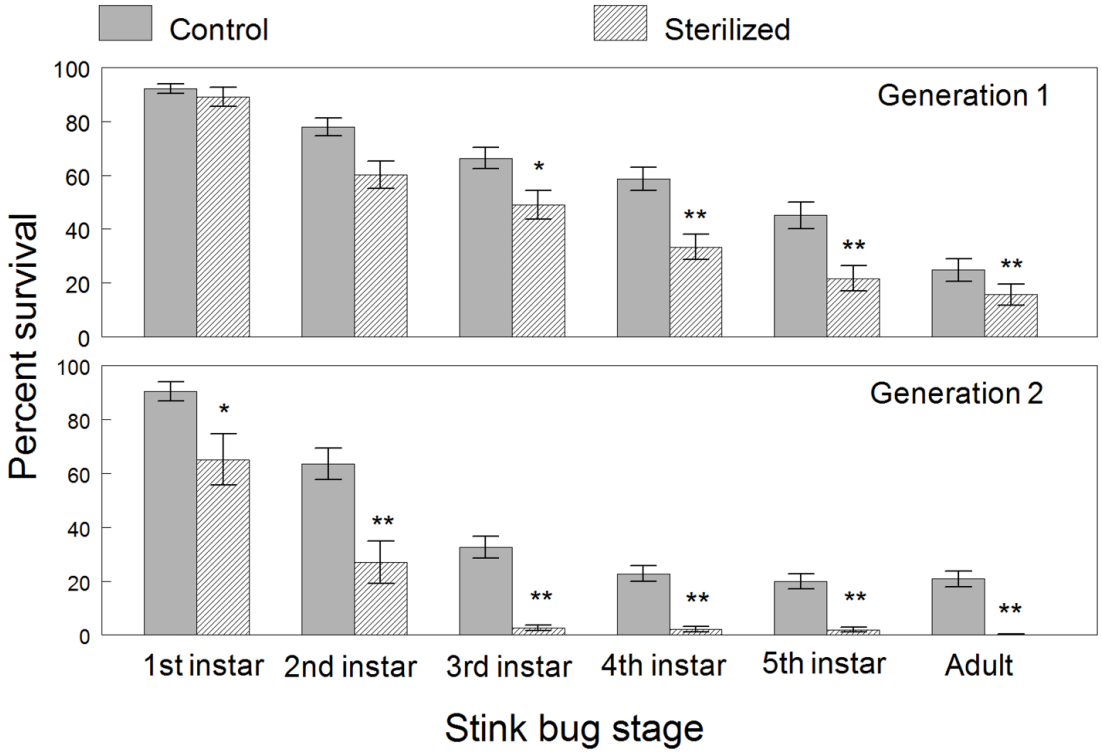

Figure 2.2. Percent survival from egg hatch to the peak density of each developmental stage of *H. halys* during two successive generations. Means (± SEM) are plotted for first generation cohorts reared from sterilized and untreated control eggs and for subs.

Table 2.3. Survivorship by life stage for both generations (Two-way ANOVA with percent survival as the dependent variable, egg mass treatment (T), generation (G), and the interaction (T x G) as fixed factors, and position in the rearing chamber as a random blocking factor).

	First generation survivorship		***Second generation survivorship***					
Life Stage	***Mean % control***	***Mean % sterile***	***Mean % control***	***Mean % sterile***	***Effect***	***Df***	***F***	***P***
1st	92.3 ± 1.66	89.3 ± 3.53	90.5 ± 3.54	65.2 ± 9.52	T	1, 71	7.92	0.006
1st					G	1, 71	4.31	0.042
1st					T x G	1, 71	5.92	0.018
2nd	78.0 ± 3.32	60.3 ± 5.01	63.5 ± 5.75	27.0 ± 7.97	T	1, 71	22.96	<0.001
2nd					G	1, 71	18.73	<0.001
2nd					T x G	1, 71	3.62	0.061
3rd	66.4 ± 4.01	48.9 ± 5.36	32.6 ± 4.04	2.7 ± 1.09	T	1, 69	39.65	<0.001
3rd					G	1, 62	77.73	<0.001
3rd					T x G	1, 69	5.82	0.019
4th	58.6 ± 4.38	33.4 ± 4.76	22.8 ± 2.87	2.2 ± 1.05	T	1, 69	58.54	<0.001
4th					G	1, 70	112.5	<0.001
4th					T x G	1, 69	0.71	0.40
5th	45.2 ± 4.99	21.6 ± 4.71	19.9 ± 2.83	2.0 ± 0.93	T	1, 69	63.5	<0.001
5th					G	1, 70	83.49	<0.001
5th					T x G	1, 69	0.38	0.54
Adult	24.8 ± 4.16	15.7 ± 3.98	20.8 ± 2.90	0.3 ± 0.26	T	1, 69	57.96	<0.001
Adult					G	1, 70	42.25	<0.001
Adult					T x G	1, 69	11.45	0.001

Parameters of fecundity and egg production

Overall, including cage reared and PCR stored adults, Control Block A produced 38 females and 29 males, and Control Block B produced 20 females and 22 males. Sterile Block A produced 26 females and 24 males, and Sterile Block B produced 12 females and 14 males. Due to the longer developmental times of cohorts reared from surface sterilized eggs, adults were produced later and the population peaked in the rearing cages later than the control cohorts (Figure 2.3). In total, 46 females from the control group of generation 1 produced 73 egg masses (1.59 per female), while 29 females from the sterilized group produced 15 egg masses (0.52 per female). Females of the control group took an average of 15.5 days to begin laying eggs, while the sterilized group took 25 days to begin oviposition (Table 2.4). All egg masses collected from generation 1 that were not assigned to second generation cohorts were reared individually until they hatched. Data on the number of eggs per mass, days to hatch, and percent hatch grouped by treatment were compiled for these egg masses together with eggs assigned to second generation cohorts. ANOVA results showed no significant difference in hatch time between the sterilized and control treatments but females reared from the sterilized group of generation 1 produced egg masses that had 38% fewer eggs and significantly lower hatch rates compared to the egg production of control females (Table 2.4).

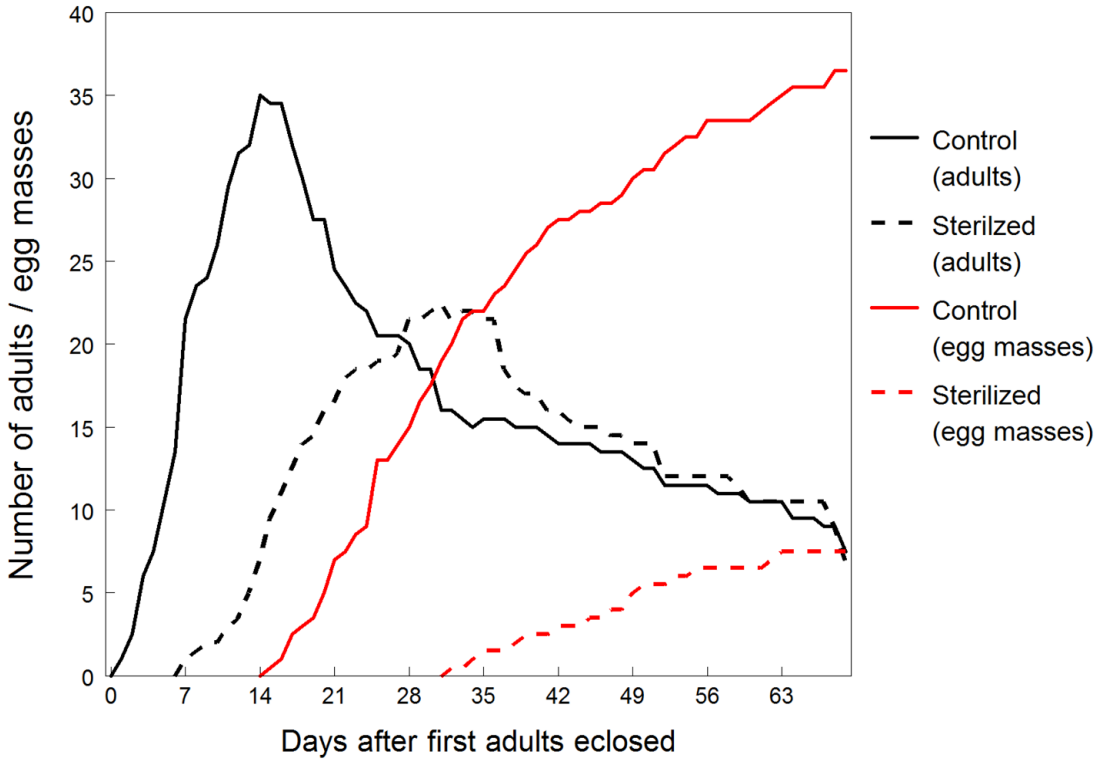

Figure 2.3. Cumulative number of generation 1 adults reared from sterilized and untreated control eggs and their egg mass production plotted over days after the first adults had eclosed.

Table 2.4. Effects of surface sterilization on the fecundity of first generation adults and on the development and hatchability of second generation egg masses of *H. halys*.

Parameter	***Control***	***Sterile***	***df***	***F***	***P***
Time to first egg mass (days)	15.5 ± 1.5	25 ± 0	-	-	-
Mean #egg masses/female	1.58 ± 0.011	0.473 ± 0.098	-	-	-
Mean # eggs/mass	27.77 ± 0.01	17.13 ± 2.05	1, 85.3	129.52	<.0001
Mean % hatch	94.43 ± 1.30	64.95 ± 9.46	1, 85	33.44	<.0001
Mean development time (days)	6.42 ± 0.08	6.50 ± 0.11	1, 83	0.18	0.6735

Movement behavior of first instars

First instar nymphs of generation 1 were observed each day for differences in their presence on and movement away from the egg mass. Similar to the methods

used by Hosokawa et al. (2008), the percentage of wandering nymphs was defined as the number of live nymphs not touching the leaf disc on which the egg mass was laid divided by the total number of live nymphs times 100. Differences between the control and sterilized treatments were analyzed using a two-way ANOVA, with each observation day treated as repeated measure fixed factor. Significant treatment differences in wandering behavior were observed but were not the same among the five days of observation, as evident by a significant interaction effect (Table 2.5). Significantly more nymphs wandered off the control egg masses during days 4 and 5 than did the nymphs on sterilized masses (Figure 2.4). At five days after egg hatch, 67% of the control nymphs exhibited wandering behavior in comparison to the 36% of nymphs hatched from sterilized eggs.

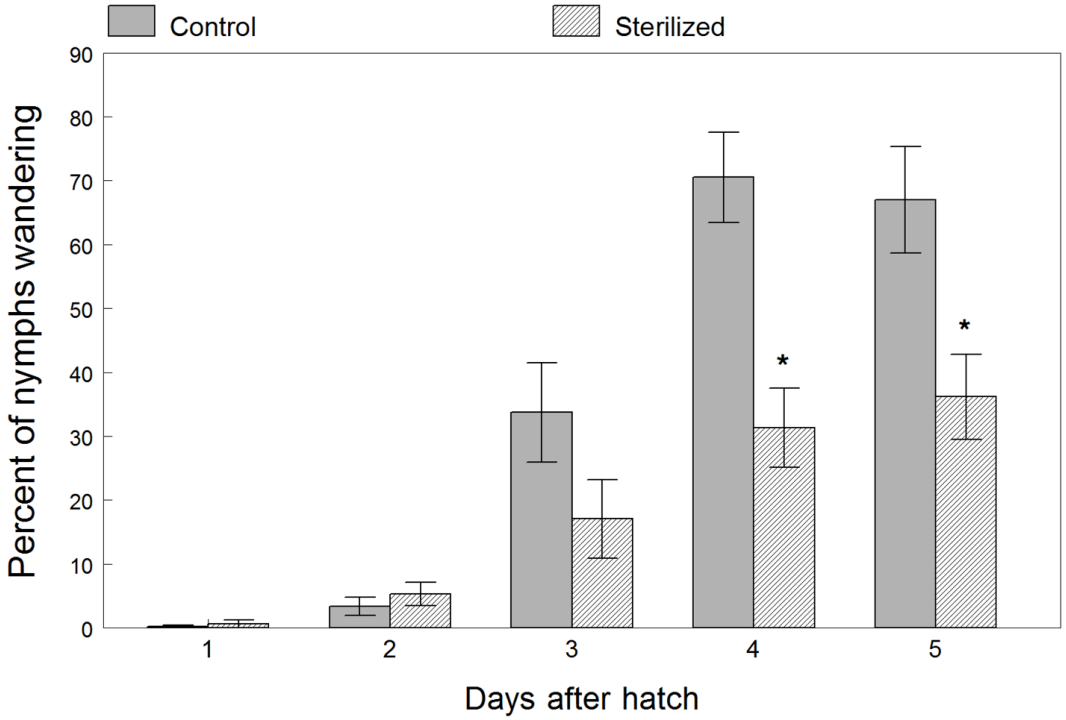

Figure 2.4. Mean percentage (± SEM) of first instars wandering away from sterilized and untreated control egg masses. An asterisk over each pair of means for each observation day indicates a significant difference ($P < 0.05$).

Table 2.5. **T**wo-way ANOVA with percent wandering behavior as the dependent variable, egg mass treatment, day of observation after hatch, and the interaction (T x G) as fixed factors, position in the rearing chamber as a random blocking factor, and adjustment made for day as repeated measure.

Effect of sterilization on wandering	***Df***	***F***	***P***
Treatment	1, 1	129.52	<.001
Day	4, 168	33.44	<.001
T x D	1, 168	0.18	<.001

Discussion

Results of this study provide direct evidence that *H. halys* is negatively impacted when its egg masses are surface sterilized, and this impact is significant in the first generation and manifests dramatically in the subsequent generation. In the first generation, developmental time and survivorship of treated cohorts were significantly affected during third instar development through to the adult stage. In the second generation, less than ten individuals collectively from the 15 egg masses laid by first generation adults of the sterilized group survived past the second instar, and of those only one successfully molted to adulthood. Adults from the sterilized treatment group exhibited longer pre-oviposition periods, produced fewer egg masses, had significantly smaller clutch sizes, and the hatch rate and survivorship of those eggs were significantly reduced. Taken together, this suggests that the overall fitness of *H. halys* is highly dependent on the presence of gut symbionts. These findings are consistent with the results reported by Prado and Almeida (2009a) on the role of gut symbionts in the stink bug *C. hilaris*. Published studies have shown that aphids rely on gut symbionts to produce certain amino acids for normal development (Douglas 1998, 2006), and the physiological role that gut symbionts play in their host has also

been reported in several heteropteran groups (Abe et al. 1995; Fukatsu and Hosokowa 2002; Prado and Almeida 2009a).

Noteworthy is the fact that the control cohorts of nymphs in the second generation experienced significantly longer developmental times and lower survival than control cohorts in the first generation. Although the rearing conditions were similar, the difference is likely due to the source of egg masses collected for the study and the different ways that they were selected for each generation. Generation 1 cohorts were selected at random from all egg masses collected on one day from several laboratory colony cages of adults which were close in age and exhibiting peak oviposition. For generation 2, egg masses were selected over several weeks from a much smaller pool of first generation adults reared from the control cohorts. We notice that egg mass production rises over a few weeks, peaks, and then decreases in our adult cages for *H. halys*. This seems to correlate to the age of the females in the cage. Egg production starts off slowly from newly reproductive females, which then increases as the adult's mature, but then tapers off at a certain point as the adults get to a certain age. The egg masses of generation 1 most likely came from middle aged females laying at their peak as opposed to the generation 2 egg masses which came from females representing different ages.

Observations were made during the first 5 days after hatch of the first generation eggs to see if treatment affected wandering behavior of the first instars. Hosokawa et al. (2008) not only draws attention to the lack of research on possible host behavior modification by a symbiont in heteropteran mutualistic relationships, but goes on to support the occurrence of this phenomenon in the species *Megacopta*

punctatissima (Plataspidae). They reported a negative correlation between the supply of symbiont available and the number of wandering nymphs. In this study, the opposite behavior occurred in *H. halys*. Nymphs hatching from untreated egg masses wandered off the mass more quickly and at a higher frequency over the five days after hatch, whereas nymphs from surface sterilized eggs tended to stay on the egg mass longer. More research needs to be done to fully understand the behavior modification in nymphs when the symbiont is absent, but it is plausible that *H. halys* nymphs once adequately inoculated will move off the egg mass, while nymphs hatching from eggs without the symbiont will continue to probe longer.

Two symbionts were sequenced from the gut of *H. halys,* although the primers used in PCR failed to detect the *Wolbachia* symbiont. It is possible that the primers were sequenced from adults that carried a different strain of *Wolbachia*, or it is not present in colony individuals. More work on this symbiotic relationship is necessary. We instead focused on *P. agglomerans* due to its confirmed extracellular presence on unhatched egg masses and in the gut of colony adults. Although egg masses from the colony tested positively for *P. agglomerans,* the qualitatively weaker gel bands detected were likely due to lower quantity of the symbiont on the egg surfaces compared to levels in the gut of adults. This notion is supported by symbiont studies performed on the Mediterranean fruit fly. The fruit fly is colonized by the symbiont *P. agglomerans*, which forms a bio-film in the gut of the fly. It migrates to the ovaries and coats the apical ends of the eggs with the same bio-film in females, albeit in a lower quantity than in the gut (Lauzon et al. 2009). Pentatomids are described as smearing gut symbionts on their eggs, but it may be possible that a similar mode of

action to the fruit flies is occurring. More work on this mechanism is needed. Detection of *P. agglomerans* in generation 1 adults was successful and the inoculation rate in the control group was significantly higher than that of the sterilized group. Despite the difference between treatment groups, there was evidence that the symbiont was not completely removed from the sterilized egg masses. One possible explanation may be attributed to the selection of adults sampled for DNA extraction. We unintentionally biased the selection towards the first individuals to molt to adulthood, thus these adults were likely the most fit individuals (they developed faster) and may have obtained symbionts as nymphs if any were present on the egg mass. For future studies, adults for DNA extraction should be selected across the entire population of each replicate cohort to obtain a more representative estimate of symbiont presence and to determine if time to adulthood correlates with symbiont inoculation.

Further research is needed to investigate environmental factors that affect the survival of the gut symbionts as well. It has been shown that high temperature can negatively impact symbiont survivorship and thus host life history parameters, as seen in *M. histrionica* and *C. hilaris* (Prado et al. 2010). Preliminary studies are showing similar trends in *H. halys* when eggs are exposed to high temperatures. Abiotic factors like temperature may prove to limit the colonization capabilities of *H. halys* in certain parts of the US.

This is the first documented evidence that removal of symbionts from the surface of the egg mass results in detrimental effects on *H. halys* life history and fecundity parameters. This may have important implications in the management of

this invasive pest. For instance, the high degree of reliance on the symbiont may be a weak link in its life history that could be exploited in field populations by a foliar application of a sterilizing agent targeting the eggs or through transgenic plant delivery of a specific antibiotic that interferes with the normal functioning of the symbionts in the gut of *H. halys*.

Acknowledgements

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Chapter 3: The impact of temperature stress on both brown marmorated stink bug symbiont acquisition and symbiont retention

Abstract

Many insects depend on gut microbes for survival. Although these microbes often provide a multitude of benefits, it has been suggested that they may also put constraints on their hosts. The brown marmorated stink bug, *Halyomorpha halys* (Stål), is reliant on a beneficial gut symbiont for proper development like many other stink bug species. In previous studies using the stink bugs *Nezara viridula, Chinavia hilaris,* and *Murgantia histrionica*, temperature increases were shown to deplete the symbionts within the bugs, indicating symbiont heat intolerance. In order to determine the impact of temperature stress on *H. halys* symbiont survival, the symbiont was exposed to temperature stress during two scenarios: first while it was present on the egg mass surface before the nymphs hatched, and then once it had colonized the gut of *H. halys*. For the egg mass study, egg masses laid on plants were exposed to growth chamber temperature fluctuations mimicking normal, above average, and extreme summer conditions for Maryland until nymphal hatch. Although there was a direct effect on hatch rate under the extreme conditions, no effect on hatch was seen between the normal and above average conditions. There was slightly lower survival to adulthood in the above average condition when compared to the normal condition despite no difference in initial hatch rate, but most adults screened from all three conditions tested positively for the symbiont, indicating that the symbiont was able to survive on the egg mass to some degree under all three

conditions. A follow up study showed no difference in inoculation rates by egg mass between the normal and above average conditions at the second instar, indicating similar symbiont survival rates at least between those two conditions. For the gut study, control *H. halys* nymphs (allowed to acquire their symbiont) and nymphs from sterilized egg masses were reared at 25 versus 30° Celsius. Fewer control bugs reared at the higher temperature retained their symbiont to the adult stage, and they also exhibited lower survival, longer development, and almost no egg mass production. Bugs from sterilized egg masses showed no differences in survival or fecundity between the two temperature treatments, but always performed more poorly than the controls within their respective temperature treatment. These results do not support the hypothesis that heat stress impacts survival of the symbiont while on the egg mass, but does support the hypothesis that prolonged exposure to heat stress can impact symbiont survival once it is within *H. halys*.

Introduction

The invasive brown marmorated stink bug, *Halyomorpha halys*, is a major agricultural and nuisance pest in the U.S. (Leskey et al. 2012a). Like other members of the Pentatomidae, *H. halys* harbors a gammaproteobacteria, *Pantoea carbekii*, in special invaginations of the gut (Bansal et al. 2014) and is dependent on the symbiont for successful development, survival, and fecundity (Taylor et al. 2014). These obligate bacteria are passed on from mother to offspring in a secretion located underneath a coating deposited on the eggs during oviposition (Kenyon et al. 2015). The first instar nymphs, upon hatching, become inoculated by immediately probing the egg chorions to acquire the gut symbiont. When newly hatched nymphs are

prevented from obtaining the symbiont bacteria, they suffered lower survival, longer developmental times, and lower fecundity as adults (Taylor et al. 2014). Since the overall fitness of *H. halys* is tied to the success of its symbiont, it is likely that conditions stressful to the symbiont will have negative repercussions for the stink bug itself.

Symbionts, especially obligate symbionts, often have physiological characteristics that make them potentially prone to heat stress, including fragile membranes due to cell surface protein loss, unstable protein folding, thermolabile structural rRNAs, and AT- rich DNA sequences (reviewed in Wernegreen 2012). In the literature, studies have reported symbiont loss at higher than normal temperatures in a number of insects. Cockroaches lost their primary symbionts at 39° C after a few weeks (Sacchi et al. 1993); the cereal weevil *Sitophilius oryzae* lost its primary symbiont at 35° C after about a month (Heddi et al. 1999); and two ant species in the genus *Camponotus* exhibited symbiont depletion when subjected to heat treatment peaking at 37.7° C per day over the course of four weeks (Fan and Wernegreen 2013). Short term heat stress can reduce levels of symbiont in the citrus mealybug *Planococcus citri* (Parkinson et al. 2014), and the primary symbionts in the aphid genus *Buchnera* are reduced under heat stress as well (Buchner 1965; Ohtaka and Ishikawa 1991; Montllor et. al 2002; Russell and Moran 2006), although the presence of secondary symbionts seems to be able to mediate heat stress on the pea aphid (Montllor et. al 2002; Burke et al. 2010). This heat stress effect has also been shown in three species of stink bugs, *Nezara viridula*, *Chinavia hilaris*, and *Murgantia*

histrionica, which experienced symbiont loss within two generations when temperatures were increased from 25 to 30° C. (Prado et al. 2009; Prado et al. 2010).

H. halys may be subject to similar adverse effects on its gut symbionts if exposed to higher than normal temperatures. Studies conducted by Venugopal et al. (2016) reported that temperature is the most important factor that influences *H. halys* population abundance and distribution at large spatial scales in the mid-Atlantic region. They further reported that stink bug densities were lower in localities with higher temperatures during July. It is unknown whether this temperature effect is due to stresses placed directly on *H. halys* or indirectly on its symbiont, but it has been suggested that host fitness reductions at higher temperatures may be tied to symbiont loss or malfunction (Prado et al. 2010). Reported here are studies to determine if temperature stress impacts symbiont acquisition and retention and ultimately reduces the fitness of *H. halys.* To test the hypothesis that higher temperatures will reduce survival of the symbiont while on the egg mass surface, egg hatch rate, nymphal survival, and symbiont acquisition were assessed after egg masses were exposed to a range of temperature conditions. To test the hypothesis that higher temperatures will reduce survival of the symbiont once it has successfully colonized the insect, egg masses were reared under normal temperature conditions until nymphs hatched and acquired their symbiont, after which nymphs were exposed to different temperature conditions and assessed daily for survival and developmental time to adulthood. In both studies, the retention rates of the symbiont were determined by PCR analysis.

Materials and Methods

Sources of insects

H. halys adults were collected from field sites throughout the summers of 2013-15 at two University of Maryland Research and Education facilities (Beltsville and Keedysville, MD) and maintained in a laboratory colony for egg production. Adults were reared in mesh cages (60 x 30 x 35 cm) on 3-week old potted green bean plants, *Phaseolus vulgaris* L., and fed excised bean pods and raw sunflower seeds. The laboratory colony provided egg masses for all experiments, except as noted in experiment 3, when additional egg masses were provided by the laboratory colony at the University of Kentucky.

DNA extraction and PCR analysis

Depending on the experiment, whole abdomens of adults or whole nymphs were prepared for DNA extraction using a Qiagen DNEasy Blood and Tissue Extraction Kit. PCR and gel electrophoresis were used to determine presence or absence of the symbiont in each adult, using the following ~930bp primers specific to the symbiont *P. carbekii* (Figure 3.1) (Bansal et al. 2014):

Forward: GCATATAAAGATTTTACTCTTTAGGTGGC

Reverse: CTCGAAAGCACCAATCCATTTCT.

Any indication of a distinct rectangular gel band, no matter how faint, was considered ‘positive’ for all samples.

In experiment 1, 12 adults from each treatment were chosen for PCR analysis from across the entire adult eclosion period to avoid potential biases. Taylor et al. (2014) reported that symbiont acquisition may be more complete in the earliest adults to eclose because they are likely more fit individuals.

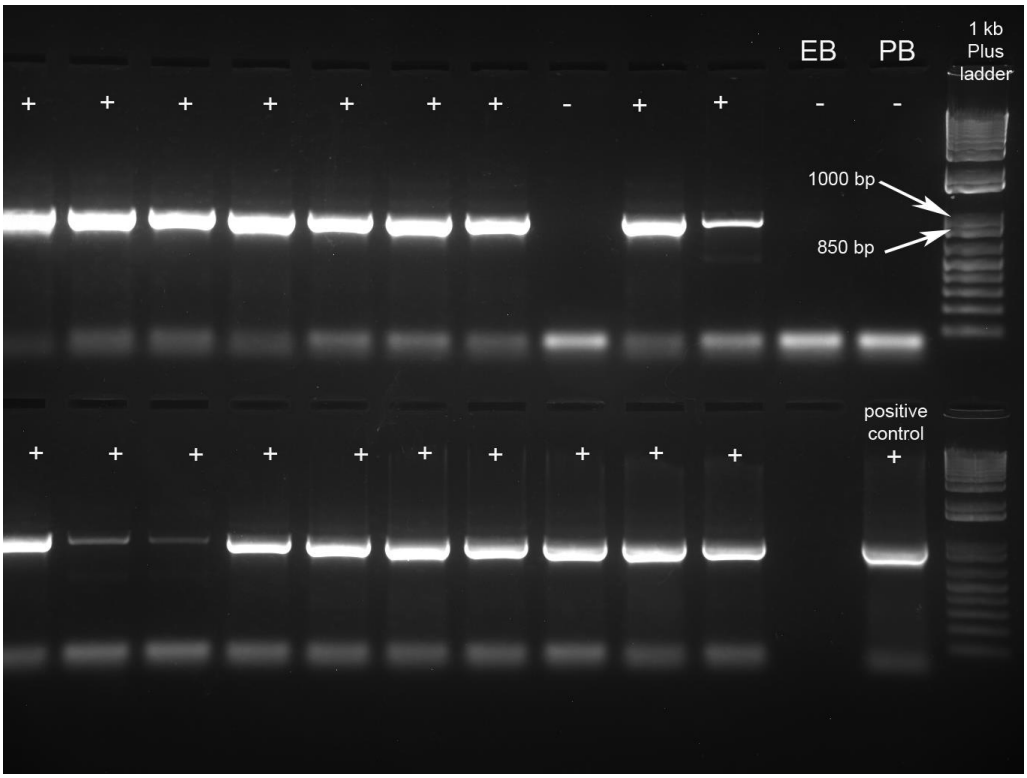

Figure 3.1. Example of gel electrophoresis with PCR products using *H. halys* second instar DNA and *P. carbekii* primers. Nineteen of the 20 nymphs from the normal temperature treatment and the positive control sample tested positively for the symbiont. Gel band amplification occurred at the appropriate location on the DNA ladder for all samples (between the 850 and 100 bp ladder bands), with no amplification in the extraction blank (EB) or PCR blank (PB). Note that there were differences in gel band intensity.

In experiment 2, because nymphal survival was lower and more variable across both treatments when compared to experiment 1, only nymphs from a selected subset of egg masses from both treatments were chosen for PCR analysis. For more consistency comparing symbiont acquisition, five nymphs were randomly selected from 12 egg masses that: 1.) Had hatch rates greater than 60%, and 2.) Had more than 50% of the nymphs surviving from first to second instar.

In experiment 3, 10 stored adults were chosen for PCR analysis from each of the four temperature and source location combination cages that originated from unsterilized egg masses. Because survival to adulthood was much lower from egg

masses that were sterilized, a total of only 28 adults were used from cages originating from sterilized egg masses (8 from MD 25°C cages, 4 from KY 25°C, 10 from MD 30°C, and 6 from KY 30°C). Because many samples tested negatively for symbiont presence, the following stink bug mitochondrial DNA 147bp primers (Kumar and Dunning-Hotopp, unpublished data) were used to confirm the quality of the DNA samples (Figure 3.2) which could be impacted by the high temperature treatment or post mortem adult collection:

Forward: CGAATCCCATTGTTTGTGTG

Reverse: AGGGTCTCCTCCTCCTGATG.

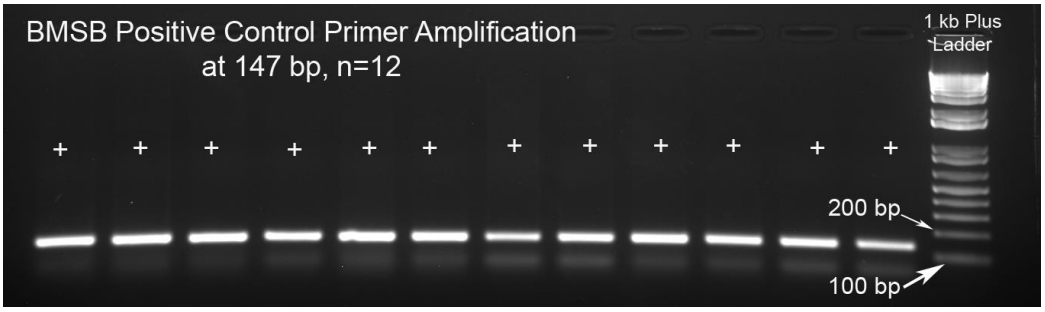

Figure 3.2. Example of gel electrophoresis with PCR products using *H. halys* adult DNA and mitochondrial DNA primers; only two of the 12 sterile adults screened from the 25 degrees Celsius treatment in the post inoculation study tested positively for the symbiont in a previous analysis, but those same DNA samples all tested positively for stink bug mitochondrial DNA above, verifying sample quality. Gel band amplification occurred at the appropriate location on the DNA ladder (between 100 and 200bp ladder bands) with no amplification in the extraction blank or PCR blank (not shown).

Statistical analysis

All analyses were performed using the mixed procedure of ANOVA (SAS Institute Inc. 2008), with individual egg masses treated as the replicate unit. For experiments 1 and 2, data per egg mass were summarized as percent of hatched eggs, percent survival by instar stage, and percent of nymphs or adults inoculated with the symbiont. The former two variables were analyzed as a one-way ANOVA (PROC MIXED), to test for a temperature treatment effect. A two-tailed Fishers Exact Test

was used to analyze for significant differences in the proportion of nymphs or adults with the symbiont. All egg masses were used for the hatch rate and survival analyses, but only a subset of nymphs or adults reared from egg masses were used for the PCR data analysis.

Data in experiment 3 were summarized as percent of hatched eggs survival by instar, developmental time from egg hatch to the peak density of each instar, egg mass production per day, and percent of adults inoculated with the symbiont. Egg hatch, nymphal survival, and development time were each analyzed as a two- way ANOVA to test for the main and interaction effects of temperature and egg mass treatment. The source of the egg masses was treated as a blocking factor. Differences in in the proportions of adult symbiont inoculation were analyzed using a Chi squared test.

Materials and Methods: Experiment 1 – Effects of temperature exposure during egg development on *H. halys* fitness to the adult stage

The objective of experiment 1 was to assess effects on egg hatch rate, nymphal survival, and symbiont presence in the adults after exposure to different temperature treatments only during egg development. With this short exposure, any heat stress effects would be manifested in the unhatched *H. halys* embryos and/or the symbiont on the egg mass surface.

Egg mass treatments

Potted plants with a total of 30 egg masses laid within 24 hours were collected on one day from the laboratory colony, and randomly split into three groups of 10. Plants with more than one egg mass were used, as long as the egg masses were on

separate leaves. Each group of plants was assigned to and placed into one of three environmental chambers. Each chamber was programmed to simulate the diurnal temperature patterns of a normal, above average, or extreme summer day in Maryland with average daily temperatures of 24, 27, and 31.5°C, respectively. Weather readings from the Central Maryland Research and Education facility at Beltsville, MD were used to determine the diurnal changes in temperature and associated humidity levels for each chamber. Each chamber had high intensity lighting for plant growth on a 16:8 (L: D) diurnal cycle and was programmed with multiple temperature and humidity set time points, as given in Table 3.1. Each environmental chamber was limited to only eight setpoints, of which sets of four points defined either the target temperatures or relative humidity. A ramp step programmed each chamber to increase or decrease linearly over the time between one setpoint to the next, as depicted in Figure 3.3.

Table 3.1. Setpoints for temperature, relative humidity, and light parameters for each of the three environmental treatments.

	Time of Day	**Temperature (°C)**	**% Humidity**	**# lights on**
Normal	2 a.m.	19.5	85%	0
	6 a.m.	20	65%	2
	2 p.m.	30	65%	2
	10 p.m.	23	65%	0
	Time of Day	**Temperature (°C)**	**% Humidity**	**# lights on**
Above Average	2 a.m.	22.2	90%	0
	6 a.m.	21.1	50%	2
	2 p.m.	34.4	50%	2
	10 p.m.	22.2	65%	0
	Time of Day	**Temperature (°C)**	**% Humidity**	**# lights on**
Extreme	2 a.m.	26.7	90%	0
	6 a.m.	25	65%	2
	2 p.m.	36	40%	2
	10 p.m.	27	65%	0

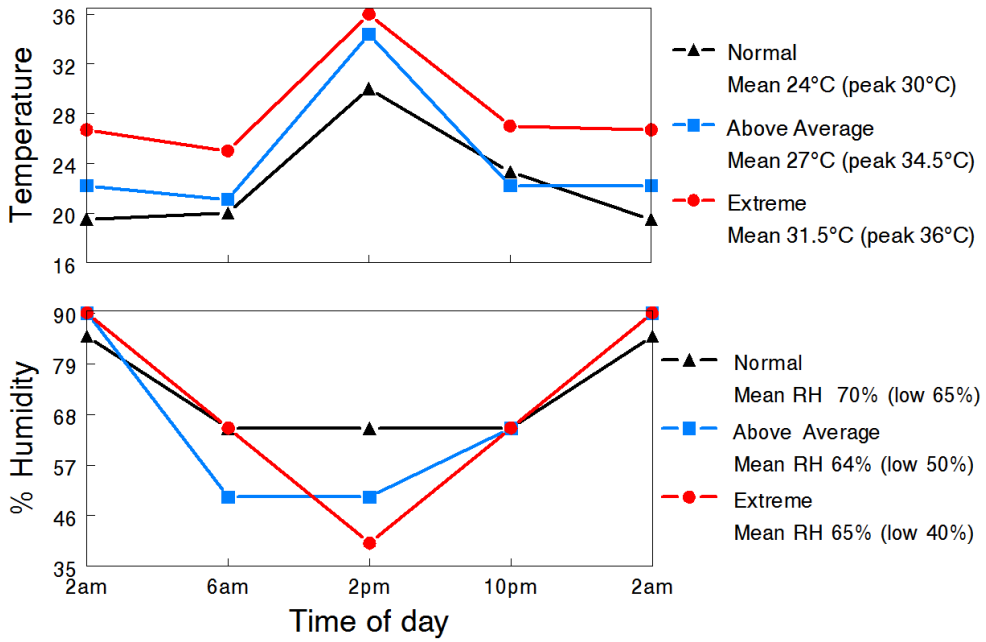

Figure 3.3. Diurnal pattern of temperature and relative density for the three environmental treatments (normal, above average, and extreme). Top graph shows temperature fluctuations for one 24 hour period. Bottom graph shows humidity fluctuations for one 24 hour period.

Nymphal rearing

Egg masses were examined daily until hatch, at which time hatched egg masses, each attached to a 15mm diameter leaf disk, were removed from the chamber. Each egg mass was placed onto a fresh excised leaf terminal of a bean plant inserted into a 15 mL floral water pick, and reared under optimal rearing conditions (25°C, 65-75% humidity, 16 h L:D diurnal cycle) in a clear plastic deli container (16x14x5 cm) with a screened lid. A bean pod was placed in each container with the leaf terminal, and both were replaced as needed to provide a fresh food supply. The content of the container was misted daily to provide a water source for the developing nymphs. Data were recorded per egg mass on the number of hatched eggs on the first day and on the number of nymphs of each stage observed daily. Once a nymph

reached adulthood, it was killed and frozen in 100% ethanol for PCR symbiont analysis.

Materials and Methods: Experiment 2 – Effects of temperature exposure during egg development on *H. halys* fitness to the second instar

This experiment was basically a duplication of experiment 1, but with a greater number of replicate egg masses exposed to the more commonly occurring daily normal and above average temperature conditions. Results from experiment 1 clearly indicated that the extreme temperature treatment resulted in significant adverse effects on egg hatchability and nymphal survival and thus did not need to be repeated. Experiment 2 also involved an earlier assessment of symbiont acquisition because the data were recorded to the second instar only.

Egg mass treatments

Potted plants with 40 egg masses less than 24 hours old were collected from the laboratory colony over four consecutive days. One group of plants with 20 egg masses were exposed to the normal temperature treatment, while the other group of 20 were exposed to the above average treatment. Egg masses were reared under the same diurnal temperature and humidity conditions for each treatment, as described in experiment 1.

Nymphal rearing

Hatched egg masses were removed from each temperature chamber and reared according to the procedures described in experiment 1. Data were recorded on the percentage of hatched eggs and number of nymphs surviving to the second instar. As

nymphs molted to the second instar, they were killed and frozen in 100% ethanol for PCR detection of the symbiont.

Materials and Methods: Experiment 3 – Effects of temperature exposure after symbiont acquisition on *H. halys* fitness

Experiment 3 determined if temperature stress during nymphal development reduces survival of the symbiont once it has successfully colonized the insect and whether it ultimately affects fitness of *H. halys*. To test this hypothesis, egg masses were reared under normal temperature conditions until nymphs hatched and acquired the symbiont, after which nymphs were exposed to two constant temperatures and assessed daily for survival and developmental time to adulthood. As a 'positive control' for comparison, sterilized egg masses were included in the experiment along with the unsterilized eggs as the nested factor within each temperature treatment.

Egg mass treatments and nymphal rearing

The laboratory colony could not provide enough egg masses for experiment 3, so additional egg masses were supplied from a colony reared at the University of Kentucky. A total of 40 egg masses (20 from Maryland and 20 from Kentucky) were randomly assigned in equal numbers from both source colonies (10 per location) to two temperature treatments. Within each treatment group, egg masses were further divided at random into egg mass treatment subgroups (sterilized or unsterilized), with five egg masses per location in each subgroup. The egg masses in the sterilized subgroup were surface sterilized according to the methods used in Taylor et al. (2014). Each egg mass was placed on a bean leaf terminal with the water pick and reared in a separate deli container held in a 25°C environmental chamber with a 16:8

(L:D) h diurnal cycle until hatch. The constant temperature of 25°C was considered optimal rearing conditions for egg development without any temperature stress on the symbiont deposited on the egg mass surface. Once an egg mass hatched, the deli container was then moved to its assigned environmental chamber set at the specific treatment designation of 25°C or 30°C. Nymphal development was monitored daily to record survival and the number of individuals reaching each instar stage.

Adult rearing

At this stage of the experiment, it was not possible to maintain breeding cohorts of adults per replicate egg mass, so eclosed adults were pooled together and reared in eight mesh cages according to combinations of their respective temperature treatment group (25°C or 30°C), egg mass treatment (sterilized or unsterilized), and source location (MD or KY). Each cage was stocked with potted green bean plants as an oviposition substrate, and a suspended feeding platform provided excised bean pods and raw sunflower seeds for additional food. For each temperatures/egg mass treatments/source combination, the number of adults introduced as they eclosed and their mortality were tracked and recorded daily. Dead adults were individually stored in the -20° F freezer in 100% ethanol for PCR detection of the symbiont. Egg masses were collected and recorded daily to measure fecundity over time.

Results and Discussion

Effects of temperature exposure during egg development

Neither the normal nor above average temperature treatments had any detectable effect egg hatchability in experiment 1 (Figure 3.4). Mean levels of 96.2% ±3.05 and 96.0% ±2.15, respectively, were statistically similar and comparable to

those observed in other studies of egg development at a constant 25° C (Nielsen et al. 2008). Similarly, percent egg hatch was not significantly different (p=0.47) when egg masses were exposed to the normal and above average temperature conditions in experiment 2; however, mean levels ranging from 61.82 to 69.2 were lower than those in experiment 1. For egg masses exposed to the extreme temperature conditions, egg hatchability was significantly reduced by 60% (38.3% ±12.4) in experiment 1, compared to egg hatch under normal and above average temperatures ($F_{(2,27)}$=19.85, p<.001). These results suggest that the higher temperatures, even for a short period of time, had adverse effects on egg hatchability by directly impacting the developing *H. halys* embryos or possibly interfering with nymphal eclosion. The extreme temperature treatment peaked at 36°C at 2 PM which means that egg masses were exposed to temperatures above 33°C for about five hours. Using constant temperature exposures in laboratory studies, Nielsen et al. (2008) determined that egg mortality averaged around 16 % at 33°C and 100% at 35°C. Additionally, the lower relative humidity (lowest at 40%) associated with the higher temperatures could have had some detrimental effect on the eggs. Other studies (Woods and Singer 2001; Buxton 2008) have shown that dehydration of insect eggs in a low humidity environment can lead to contraction and shrinking of both the chorion and the embryo. Even if eggs hatch after exposure to low relative humidity, the neonate may not be able to eclose successfully from the chorion owing to loss of lubrication and cuticular softness (Guarneri et al. 2002).

Egg hatch was also much more variable when subjected to the higher temperatures. This variability may be related to the specific location of the egg mass

on the plant, as well as the size and physiological condition of the leaf where it is attached. Studies have shown that the conditions on the undersides of leaves are usually much cooler than the surrounding ambient temperature, and this favorable microenvironment for eggs allows some insect species to survive in areas where ambient temperature would be lethal for the eggs (Potter et al. 2009). Not only does this egg laying behavior protect the eggs from heat, but it also protects them from direct sunlight. Soybean leaf curling in the field when conditions were extremely hot and stressful for the plants have lethally exposed *H. halys* egg masses to higher temperatures (Hooks, personal communication). Such exposure to direct sunlight, even at lower temperatures, could have adverse effects on the symbiont as well as direct effects on the eggs.

Table 3.2 summarizes the percent survival based on the peak density of each nymphal instar or adult relative to the number of eggs per replicate egg mass. Survival was significantly reduced by the extreme temperature treatment at all developmental stages, except for the adults. Differences were not significant for all comparisons between the normal and above average treatments. However, individuals reared from egg masses exposed to the above average treatment showed a consistent numerical trend of lower survival at each development stage compared to individuals exposed to the normal temperature treatment. In particular, it is noteworthy that the survival to the adult stage of individuals from the above average treatment was slightly less than one half that of individuals reaching adulthood from the normal temperature treatment. Although these differences are not statistically significant, the trend suggest the possibility that temperature stress on the developing egg mass could

have indirect effects later on the fitness of later developmental stages, as seen in other insects exposed to heat stress at particular life stages such as the whitefly species *Trialeurodes vaporariorum* and *Bemisia tabaci* (Cui et al. 2008), the multicolored Asian lady beetle *Harmonia axyridis* (Zhang et al. 2014), diamondback moth *Plutella xylostella* (L.) (Zhang et al. 2013; Xing et al. 2014; Zhang et al. 2015), and brown planthopper *Nilaparvata lugens* (Stål) (Piyaphongkul et al. 2012). To examine for possible treatment effects after egg hatch, the adjusted percent survival rates depicted in Figure 3.5 are computed relative to the number of hatched eggs (or first instars) rather than the total number of eggs. Differences among treatments for the second and third instar are similar to those given in Table 3.2; however, survivorship across the developmental stages declines noticeably less for individuals that survived exposure to the extreme temperature treatment during egg development. It is possible that these trends in survival suggest possible carryover effects from the temperature-exposed egg masses on *H. halys* fitness.

Fitness after egg hatch has also been shown to be largely dependent on acquisition of the gut symbiont. It was possible that lower symbiont acquisition rates at the higher temperatures was responsible for the numerical trend of lower survival seen in the above average treatment despite the initial similarity in hatch rate and survival to the normal treatment. Experiment 2 examined whether symbiont acquisition was affected by egg mass exposure to the normal versus above average temperature treatments, and results showed no significant differences in hatch rate or survival to the second instar, or the percentage of second instars inoculated with the symbiont (Table 3.3, Figure 3.6). The evidence from experiment 2 clearly shows that

egg mass exposure to above average daily temperatures has little if any adverse effect on egg hatch, nymphal survival, and symbiont acquisition.

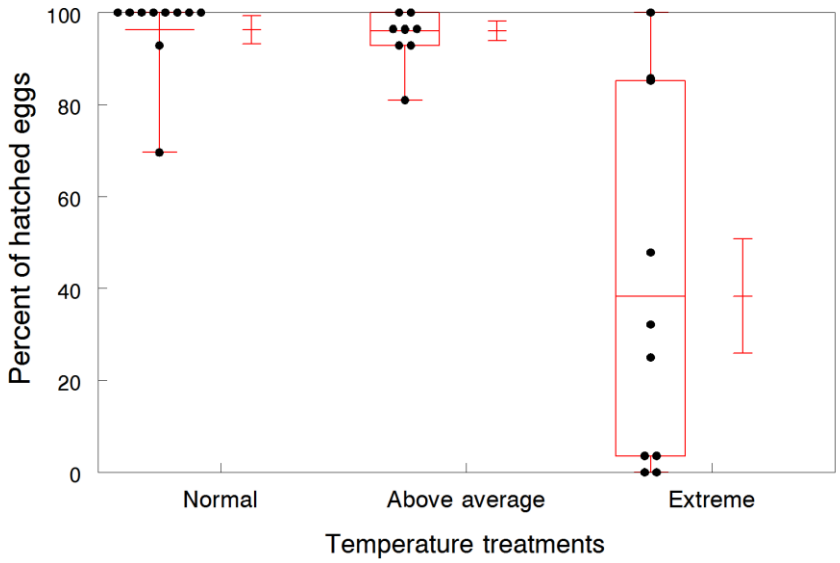

Figure 3.4. Effects of normal, above average, and extreme temperature treatments on the percentage of hatched *H. halys* eggs. Each box graph displays the range of 25th to 75th percentile of the individual replicate values. The mean (± SE) for each treatment is based on 10 replicate egg masses and given at the right of each box. Mean percent hatch of egg masses exposed to the extreme temperature treatment is significantly lower compared to that of the other two treatments (*p=0.05*).

Table 3.2. Summary of the one-way ANOVA means and statistics for percent survival per developmental stage across three temperature treatments. Percent survival is based on the peak density of each nymphal instar or adult relative to the number of initial eggs per replicate egg mass. Asterisk after means indicate that a significant difference compared to the other treatments.

Develop. Stage	**Normal temperature**	**Above average temperature**	**Extreme temperature**	**Degrees of freedom**	**F value**	***p* value**
	Mean percent survival (± SE)					
1st instar	96.2 ± 3.05	96.0 ± 2.15	38.3 ± 12.43*	2,27	19.85	<.001
2nd instar	88.9 ± 4.20	75.6 ± 7.89	23.9 ± 8.87*	2,27	22.38	<.001
3rd instar	62.6 ± 9.19	51.9 ± 7.08	11.5 ± 4.67*	2,27	13.95	<.001
4th instar	47.4 ± 8.13	39.3 ± 5.98	11.1 ± 4.72*	2,27	8.75	0.001
5th instar	29.0 ± 5.33	23.8 ± 3.85	8.9 ± 4.08*	2,27	5.46	0.010
Adult	17.5 ± 5.69	8.3 ± 3.58	5.3 ± 2.36	2,27	2.39	0.111

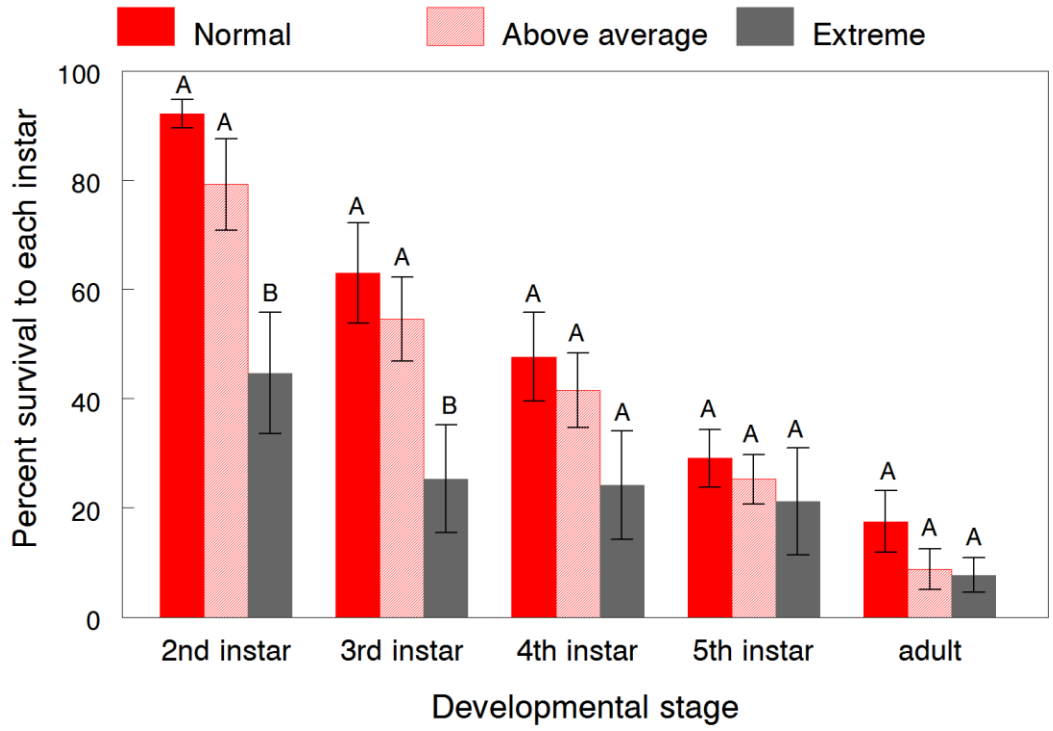

Figure 3.5. Effects on percent survival of second instars through to adult *H. halys*, following egg mass exposure to normal, above average, and extreme temperature treatments. Percent survival is based on the peak density of each nymphal instar or adult relative to the number of hatched eggs per replicate egg mass. Means within each developmental stage with the same letter are not significantly different (p=0.05).

Table 3.3. Summary of the one-way ANOVA means and statistics for percent egg hatch, survival to the second instar, and percent of second instars inoculated with the symbiont, as affected by the normal and above average temperature treatments in experiment 2.

Fitness parameter	**Temperature treatment**		**Degrees of freedom**	**F value**	***p* value**
	Normal	**Above Average**			
Mean % hatch Rate	69.2 ± 5.36	75.0 ± 5.69	1,38	0.54	0.4664
Mean % survival to second instars	61.8 ± 6.21	55.3 ± 6.89	1,38	0.49	0.4812
Mean % inoculated	80.0 ± 8.88	83.3 ± 6.89	1,22	0.09	0.7695

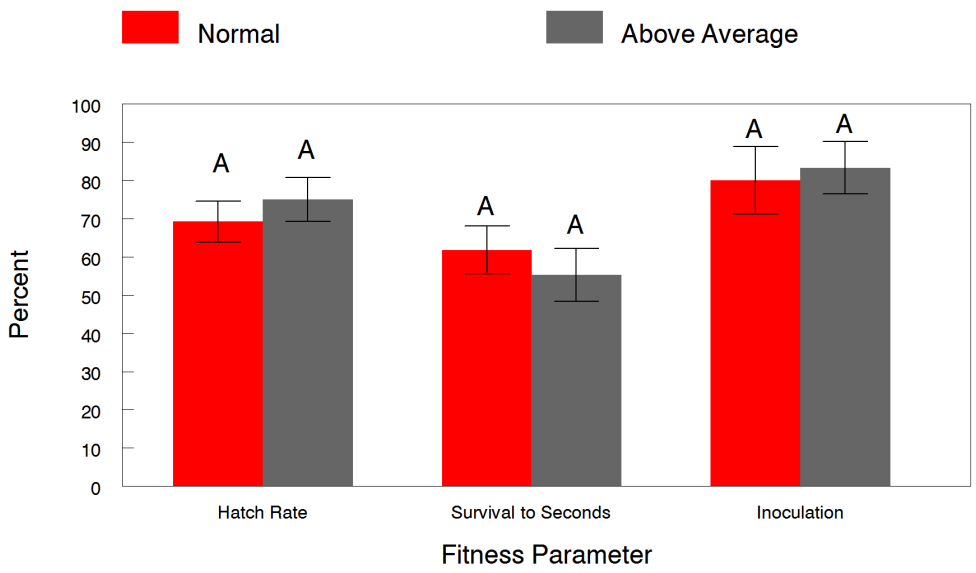

Figure 3.6. Effects on egg hatchability, survival to second instars, and percent of second instars inoculated with the symbiont, following egg mass exposure to normal and above average temperature treatments. Percent survival is based on the peak density of the second instar relative to the number of hatched eggs per replicate egg mass. Means within each fitness parameter with the same letter are not significantly different (p=0.05).

Effects of temperature exposure after symbiont acquisition

In experiment 3, both sterilized and unsterilized egg masses were reared under optimum temperature conditions until hatch, and the first instars were allowed to acquire (or attempt to acquire) the gut symbionts, after which cohorts of individuals from each egg mass treatment were reared under two constant temperatures (25 and 30°C). Two way ANOVA results showed no significant main effects of egg mass treatment or temperature or its interaction on survival of the first and second instar nymphs, but significant main effects of both factors on the third instar (Table 3.4, Figure 3.7). Significantly fewer nymphs from the sterilized egg treatment survived to the third instar (35.8% ± 3.64 SE compared to 56.7% ± 4.99 for the unsterilized treatment). Surprisingly, nymphs reared under 30°C survived better to the third instar

(54.50 ± 5.00 SE), even though 25°C was considered a more optimum temperature for rearing *H. halys* (Nielsen et al. 2008). Differences between egg mass treatments were relatively the same between temperatures, as evident by a nonsignificant interaction effect. Survival to the fourth and fifth instars was not affected by the temperature treatments but significantly reduced by the egg mass treatment. As expected, survival of nymphal cohorts reared from sterilized eggs was consistently lower within each temperature treatment. Pooled over both treatments, main effects from sterilized and unsterilized treatments respectively averaged 25.7% ± 3.66 versus 40.9% ± 4.25 for fourth instars and 18.1% ± 3.34 versus 36.8% ± 4.37 for fifth instars. Although the interaction effects at both instars were not significant, there was a consistent trend of higher survival of nymphs reared from sterilized eggs at 30°C. This would represent the opposite effect predicted if the lack of the symbiont resulted in a lower tolerance to higher temperatures. This symbiont-mediated function has been documented in aphids (Montllor et. al 2002) and whiteflies (Brumin et al. 2011) to influence thermal tolerance and a higher fitness when the host is subjected to high temperatures. By the adult stage, the interaction effect of temperature and egg mass treatment was significant ($F_{(1,32)}$=6.29, p=.017), showing a significant 34.4% reduction in survival of adults reared from unsterilized eggs at 30°C compared to those from unsterilized eggs reared at 25°C (Figure 3.7). The decrease in survival during the fifth stadium until the adult stage may be due to the loss of the symbiont at some point during the earlier instars but also partly due to a direct effect of the higher temperature on *H. halys* fitness. Nielsen et al. (2008) did show a stage-specific and generational mortality difference during *H. halys* development at 30°C compared to

25°C. However, their findings at the higher temperatures could be partly attributed to heat stress on the symbiont. Statistically, there was no treatment difference in survival of the aposymbiotic stink bugs but overall survival was significantly lower compared to the symbiotic adults, which was previously demonstrated by Taylor et al. (2014) when egg masses are chemically sterilized.

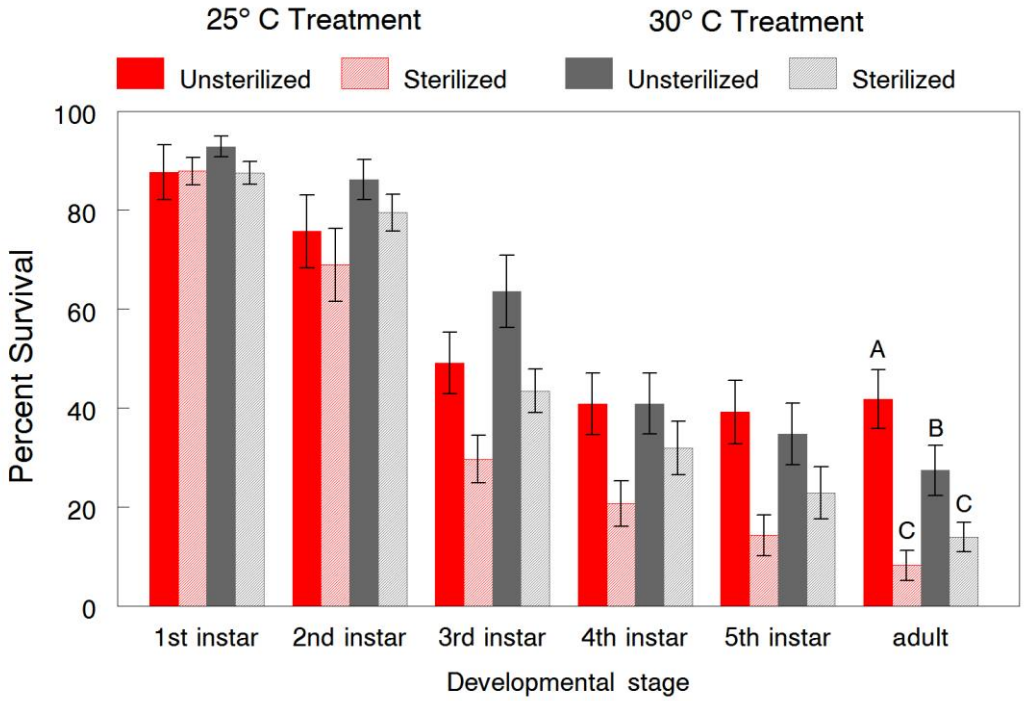

Figure 3.7. Effects on percent survival of first instars through to adult *H. halys* of cohorts reared from sterilized and unsterilized egg masses under two constant temperatures. Mean percent survival (± SE) is based on the peak density of each nymphal instar or adult relative to the number of initial eggs per replicate egg mass. Survival responses during the first and second stadia were not affected by the treatment factors. Both main effects of egg mass treatment and temperature significantly affected survival to the third instar. Only the egg treatment effect was significant for the fourth instar. Interaction means for the adult stage with the same letter are not significantly different (p=0.05).

Table 3.4. Summary of the two-way ANOVA means and statistics for percent survival per developmental stage across two egg mass treatments and two temperature treatments. Percent survival is based on the peak density of each nymphal instar or adult relative to the number of initial eggs per replicate egg mass. Interaction means for the adult stage with the same letter are not significantly different (p=0.05).

Life stage	Mean percent survival (±SE)				ANOVA statistics			
	25° C		30° C			Degrees of freedom	F value	p value
	Unsteril.	Steril.	Unsteril.	Steril.	Effect			
1st	87.6 ± 5.55	87.9 ± 2.78	92.8 ± 2.12	87.5 ± 2.34	trt	1,32.3	0.58	0.452
					temp	1,32.3	0.45	0.509
					trt x temp	1,32.1	0.69	0.413
2nd	75.7 ± 7.40	68.9 ± 7.36	86.2 ± 4.05	79.5 ± 3.68	trt	1,32.1	2.05	0.162
					temp	1,32.1	2.81	0.103
					trt x temp	1,32	0.01	0.938
3rd	49.1 ± 6.21	29.6 ± 4.82	63.6 ± 7.27	43.5 ± 4.45	trt	1,32	17.04	<0.001
					temp	1,32	5.99	0.020
					trt x temp	1,32	0.03	0.861
4th	40.8 ± 6.19	20.7 ± 4.57	40.9 ± 6.18	31.9 ± 5.43	trt	1,32.1	8.99	0.690
					temp	1,32.1	0.75	0.392
					trt x temp	1,32	0.98	0.329
5th	39.2 ± 6.44	14.3 ± 4.01	34.7 ± 6.20	22.8 ± 5.32	trt	1,32.1	13.16	<0.001
					temp	1,32.1	0.05	0.830
					trt x temp	1,32.1	1.36	0.252
Adult	41.8 ± 5.94a	8.2 ± 3.06c	27.4 ± 5.04b	13.9 ± 3.02c	trt	1,32	41.63	<.001
					temp	1,32	2.11	0.156
					trt x temp	1,32	6.29	0.017

Development time was significantly affected by both temperature and egg mass treatment but the main or interaction effects depended on the particular *H. halys* developmental stage (Table 3.5, Figure 3.8). As expected, developmental time from

egg hatch to third instars was significantly shorter at 30°C (total of 12.7 days) compared to 25°C (total of 17.5 days). However, main effect responses from third to fourth instars changed, whereby development time became more delayed in nymphal cohorts that were reared from sterilized eggs and the temperature effect was less. Irrespective of temperature, developmental time from third to fourth instars averaged 11.3 ± 0.478 days for cohorts reared from sterilized eggs compared to 8.63 ± 0.485 days for cohorts reared from unsterilized eggs. Development from the fourth to fifth instar showed a significant interaction effect between temperature and treatment. The developmental time was statistically the shortest for nymphs reared on unsterilized eggs at 25°C; however, developmental times for nymphs reared from unsterilized and sterilized eggs at 30°C were significantly longer, and this response was even more evident for nymphs reared from sterilized eggs at 25°C. This trend with temperature carried over to the fifth instar, with nymphs from both sterilized and unsterilized cohorts taking about four more days to reach adult eclosion at the higher temperature. Overall, nymphal development starting after the fourth instar showed a consistent delay at 30°C, which was contrary to the study by Nielsen et al. (2008) that reported shorter development time for late instars to reach adulthood at 30°C compared to that of 25°C. This lag in developmental time is likely due to temperature stress on the symbiont since both symbiotic and aposymbiotic nymphs were affected in the same way.

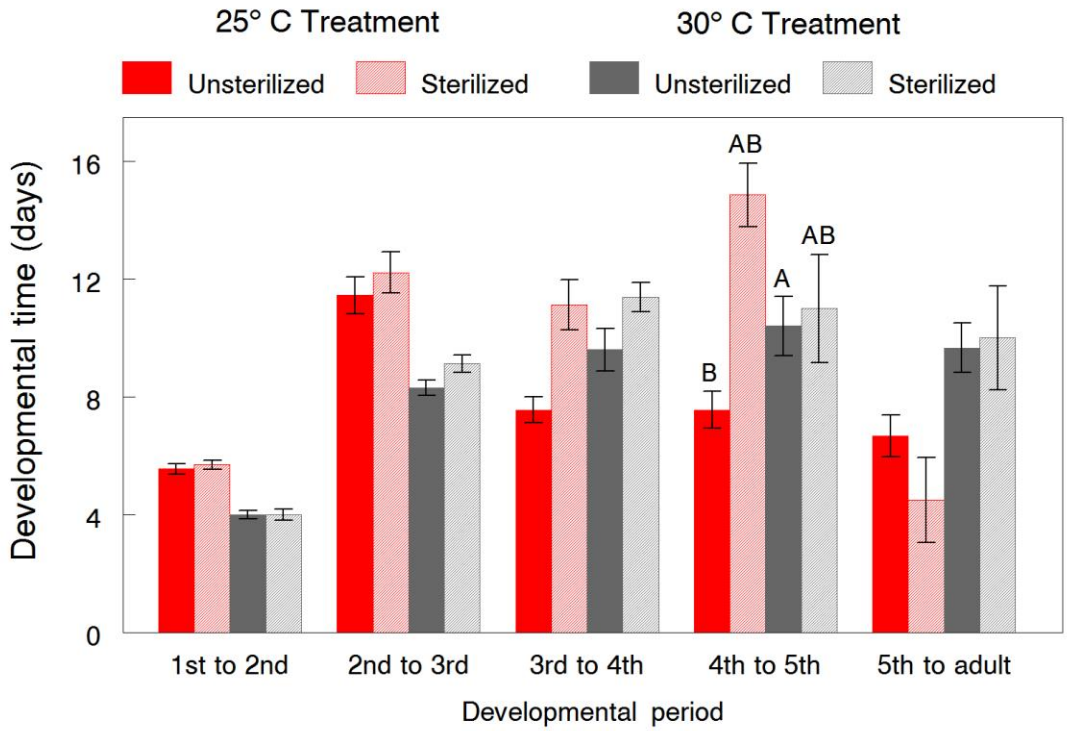

Figure 3.8. Effects on developmental time of first instars through to adult *H. halys* of cohorts reared from sterilized and unsterilized egg masses under two constant temperatures. Development time was based on the mean days between peak densities of successive nymphal instar or adult stages. Development time during the first to third instar period were significantly affected by the temperature treatment. The main effect of egg mass treatment significantly affected development from the third and fourth instar. Development time from the fourth and fifth instars was significantly affected the egg mass treatment but differences depended on the temperature, as indicated by the interaction means which were not significantly different if associated with same letter (p=0.05). Temperature significant affected development from the fifth instar to the adult stage.

Table 3.5. Summary of the two-way ANOVA means and statistics for developmental periods of first instar through to adult *H. halys* reared under two egg mass treatments and two temperature treatments. Development time was based on the mean days between peak densities of successive nymphal instar or adult stages.

Life stage period	Mean develop. time (days ±SE)				ANOVA statistics			
	25° C		30° C		Treatment factors	Degrees of freedom	F value	*p* value
	Unsteril.	Steril.	Unsteril.	Steril.				
1st to 2nd	5.6 ±0.18	5.7 ±0.15	4.0 ±0.15	4.0 ±0.19	trt	1,32	0.66	0.422
					temp	1,32	124.24	<0.001
					t x t	1,32	0.15	0.697
2nd to 3rd	11.4 ±0.63	12.2 ±0.70	8.3 ±0.26	9.1 ±0.30	trt	1,32	2.46	0.127
					temp	1,32	37.29	<0.001
					t x t	1,32	0.00	0.963
3rd to 4th	7.6 ±0.44	11.1 ±0.85	9.6 ±0.72	11.4 ±0.50	trt	1,31	16.64	<0.001
					temp	1,31	3.07	0.090
					t x t	1,31	1.88	0.181
4th to 5th	7.6 ±0.63	14.9 ±1.08	10.4 ±1.00	11.0 ±1.83	trt	1,30	11.00	0.002
					temp	1,30	0.18	0.674
					t x t	1,30	7.91	0.009
5th to adult	6.7 ±0.71	4.5 ±0.44	9.7 ±0.83	10.0 ±1.77	trt	1,24	0.62	0.440
					temp	1,24	13.24	0.0013
					t x t	1,24	1.15	0.2952

As the adults eclosed from replicate cohorts of each temperature and egg mass treatment combination, they were pooled together and reared to measure egg production per female. Since there was a noticeable difference in fitness performance of nymphs reared from Maryland and Kentucky egg masses, separate cages were established for adults from each source colony and further divided by temperature and egg mass treatments. Several fitness parameters are given in Table 3.6 showing much higher numbers of adults and egg masses produced by the unsterilized cohorts at 25° C compared to levels recorded for unsterilized cohorts at 30° C. Although it was not

possible to test statistically for differences due to data pooling across replicates, the KY adults from sterilized egg masses were slightly more fecund than MD adults, but exhibited less overall fitness compared to MD adults when reared from sterilized eggs. At 30° C, egg mass production all but ceased for adults from both source colonies and egg mass treatments. These results are in agreement with the previous study by Taylor et al. (2014) that reported significantly lower survival, longer development, and lower fecundity in aposymbiotic stink bugs.

When screened for the presence of the symbiont by treatment combination, adults from unsterilized eggs and reared at 25° C showed the highest percentage of symbiont presence with 85% testing positively. However, symbiont presence in adults reared at 30° C dropped to 35%, indicating symbiont loss at the higher temperature. As expected, symbiont presence was very low for adults from sterilized egg masses at 25° C. with 12.5% testing positively, and there was no evidence of symbiont presence at 30° C. Although stress response genes are documented for *P. carbekii* (Kenyon et al. 2015), it has been shown in the aphid symbiont *Buchnera* that differential expression of these genes is modest at best under heat stress. It is suggested that these stress response genes are present to assist with general problems that are a consequence of the symbiont's reduced genome, such as issues with protein misfolding and degradation (Wilcox et al. 2003), especially when one also considers that these stress response genes are usually constantly being expressed even when under no external stressor pressure. Future research into the changes in gene expression for *P. carbekii* might reveal similar observations.

Table 3.6. Summary of fitness performance of adult *H. halys* from two source colonies reared from sterilized and unsterilized egg masses under two temperature treatments. Data for each treatment combination are pooled across all replicate cohorts of each egg mass and given as the total number of adults and egg masses produced, fecundity per female, and days from first adult eclosion to the beginning of egg laying. ([a] No egg masses were laid by KY stink bugs reared from sterilized eggs at both temperatures, so days to egg laying could not be determined)

Treatment combinations			Total number of egg masses	Total number of adults	Mean number of egg masses per female	Days from first adult eclosion to egg laying
Temp.	Egg mass treatment	Source location				
25° C	Unsterilized	MD	98	32	3.06	15
		KY	118	25	4.72	17
	Sterilized	MD	10	8	1.25	24
		KY	0	3	0.0	[a]
30° C	Unsterilized	MD	2	23	0.09	22
		KY	2	13	0.15	21
	Sterilized	MD	1	8	0.13	18
		KY	0	3	0.0	[a]

Altogether, the results of these studies support the notion that heat stress likely impacts *H. halys* fitness both directly on the insect but also indirectly through its symbiont once it colonizes the gut, but it was not possible to separate the two effects. These results also build upon the findings of Venugopal et al. (2016) that temperature is the factor that mediates stink bug populations at large spatial scales, namely that the hotter it is, the fewer stink bugs there are. Interestingly, the source of the stink bugs resulted in noticeable differences in fitness performance as well, suggesting that future work should focus on differences in *H. halys* fitness, fecundity, and symbiont retention across different locations and temperature conditions. This information, particularly fitness performance and symbiont acquisition across the distribution range of *H. halys* might add further support to the idea that heat stress on the symbiont may be limiting its host's expansion into hotter areas of the country.

Acknowledgements

I would like to thank Peter Coffey and Veronica Johnson for their assistance with the arduous task of daily data collection and maintenance of cohorts for all three studies when I was unable to do so myself. I would also like to thank Dr. Ricardo Bessin at the University of Kentucky for sending me egg masses when our colony was not producing enough for me to complete my studies. A special thank you to Galen Dively and Charles Mitter for their assistance with reviewing and revising this work.

Chapter 4: Assessing the use of antimicrobials to sterilize brown marmorated stink bug egg masses and prevent symbiont acquisition

Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is dependent on a beneficial obligate symbiont for successful development, survival, and fecundity. The bacteria are deposited on the egg mass surface by the female, and first instar nymphs become inoculated with the bacteria by probing the egg chorions upon hatching. Targeting the bacteria exposed on the egg mass surface may prove to be a viable management strategy for the stink bug. Egg masses were surface-treated with one of six antimicrobials and surfactants to determine if exposure to these products adversely affected the fitness of newly-hatched nymphs and/or sterilized the egg mass surface to prevent nymphal acquisition of the symbiont. Egg hatch rate was significantly reduced by the streptomycin sulfate (Agri-Mycin 17) and the surfactant (Naiad) products, nymphal survival was significantly impacted by the azadirachtin (AzaGuard) and surfactant (Naiad) products, and symbiont acquisition was significantly disrupted by the azadirachtin (AzaGuard), surfactant (Naiad), and copper (Liquid Copper Fungicide) products. In a similar field study, there were no significant treatment effects on nymphal survival or symbiont acquisition, but egg hatch rate was reduced by both surfactants tested. Products with both antimicrobial effects and the ability to penetrate the coating covering the bacteria were most effective in disrupting symbiont acquisition.

Introduction

Insect-microbe associations are very common across many orders of insects, including the Hemiptera, which contains many economically important insect pests that have associations with primary and secondary symbionts (Buchner 1965). The following studies point out that many of these economically important species not only harbor symbionts, but are in fact dependent on them for successful survival due to the plethora of ways that they benefit their hosts. Economically important groups within the Sternorrhyncha and Auchenorrhyncha include leafhoppers (Auchenorrhyncha: Cicadellidae: Moran et al. 2003; Takiya et al. 2006; Wu et al. 2006; Noda et al. 2012; Ferrater et al. 2013), aphids (Sternorrhyncha: Aphididae: Douglas 1998; Oliver et al. 2003; Donald et al. 2016; Enders and Miller 2016; Zhang et al. 2016), and whiteflies (Sternorrhyncha: Aleyrodidae: Zchori-Fein and Brown 2002; Himler et al. 2011; Bing et al. 2012). Within the Heteroptera, these associations are primarily documented for the superfamily Pentatomoidea (Abe et al. 2005; Fukatsu and Hosokowa 2002; Prado et al. 2006; Prado and Almeida 2009a; Kawa et al. 2010; Bansal et al. 2014; Taylor et al. 2014). With recent improvements in genetic identification techniques, more of these relationships are being recognized and characterized (Moran et al. 2008).

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is an invasive pest from Southeast Asia (Hoebeck and Carter 2003) that has been steadily expanding its range in the U.S. since the mid-1990s (Haye et al. 2015). Originally detected in Allentown, Pennsylvania, it has become a severe agricultural pest in the Mid-Atlantic States and surrounding areas (Leskey et al. 2012a). The brown marmorated stink bug,

like many other species in the family Pentatomidae, harbors a gammaproteobacteria in the fourth region of the midgut and is dependent on this symbiont for successful development, survival, and fecundity (Taylor et al. 2014). These bacteria, described as a new species *Pantoea carbekii*, are free living in special invaginations of the gut, referred to as crypts (Bansal et al. 2014). They are passed on from mother to offspring via a coating which is secreted on the eggs during oviposition, and are located underneath and intercalated within this secretion (Kenyon et al. 2015). The first instar nymphs, upon hatching, become inoculated by immediately probing the egg chorions. Observations of depressions in the head capsule while probing indicate nymphal sucking behavior, so it is very likely that nymphs regurgitate onto the egg chorions and/or use existing moisture to take up the bacteria (C.T., personal observations). This probing behavior only lasts for a few hours, and once the nymphs have fully sclerotized the probing behavior ceases. This type of extracellular acquisition is common in other stink bug species (Otero-Bravo and Sabree 2015), and is in contrast to the method of transmission in the Auchenorrhynchan and Sternorrhynchan groups, where the bacteria are housed within special cells called bacteriocytes and passed on to the next generation transovarially (Buchner 1965; Moran and Telang 1998; Moran et al. 2008).

Because the bacteria are present on the surface of the egg mass for the duration of the pre-hatch period, they are exposed to the surrounding environment and thus may be easy to manipulate. In studies requiring aposymbiotic stink bugs to be generated, a sterilizing agent such as bleach on the egg mass was used to remove the bacteria from the surface and prevent the newly hatched nymphs from acquiring

them (Prado et al. 2006; Prado and Almeida 2009a). Although targeting or manipulating the symbiont has been suggested as a possible management strategy (Douglas 2007; Prado and Zucchi 2012), real world applications of an egg mass sterilization method are lacking in the literature. Mathews and Barry 2015 did show impacts on hatch rate and early instar survival when BMSB egg masses were treated with compost tea, but if this impacted the symbionts on the egg mass surface was not verified. Many antimicrobial products are available commercially for the management of plant pathogens, such as bacterial blight, mildew and other fungi, and these products may also have antimicrobial effects on the symbiont bacteria. Residues present on the egg masses may be inadvertently ingested by the nymphs during probing behavior and may have direct effects on nymphal fitness as well. This may have accounted for the decreases in nymphal survival when compost tea was applied to egg masses in Mathews and Barry 2015. The brown marmorated stink bug is heavily reliant on these bacteria for survival (Taylor et al. 2014), and can be viewed as a weak link in the life history of this insect. Efforts to target the bacteria themselves may lead to effective management suppression of *H. halys*.

Reported here are studies to determine if egg mass exposure to a number of commercially available products, including antimicrobials and surfactants, adversely affects the fitness of *H. halys* nymphs and/or sterilizes the egg mass surface to prevent nymphal acquisition of the symbiont. Replicate egg masses were surface-treated with each product in the laboratory and evaluated for egg hatchability, nymphal survival to the second instar, and nymphal inoculation rates. The most effective products were also evaluated by exposing egg masses to spray solutions

delivered by simulated airblast application in a peach orchard under field conditions. I hypothesized that one or more of these products would have direct effects on nymphal fitness and symbiont acquisition.

Materials and Methods: Laboratory Study

Insect culture

H. halys adults were collected from field sites throughout the summer of 2014 at two University of Maryland Research and Education facilities (Beltsville and Keedysville, MD) and maintained in a laboratory colony for egg production. Adults were reared in mesh cages (60 x 30 x 35 cm) on 3-week old potted green bean plants, *Phaseolus vulgaris* L., and fed bean pods and raw sunflower seeds. Egg masses laid on the leaves of plants were removed within 48 hours with a cork borer to produce a 15mm leaf disk with each egg mass. Egg masses collected were randomly assigned to each treatment.

Product information

Many antimicrobials and surfactants are commercially available. However, a management strategy targeting the egg mass would be more applicable in organic operations and also more achievable in orchard systems, because many fruit growers rely on antimicrobials to manage fruit rots, and *H. halys* is a major pest of tree fruit crops (Leskey et al. 2012b). Therefore the following six commercial products were chosen for evaluation: 1) Naiad (Naiad Company, Inc.), a combination of non-ionic and ionic surfactants used as a wetting agent in turf, ornamentals, and agriculture. This product and other surfactants have been shown to reduce plant disease severity by acting as an antimicrobial agent (Stanghellini 1987, 1996; Stanghellini and Miller

1997; Irish et al. 2002; Mickler 2002); 2) AzaGuard (BioSafe Systems LLC), an OMRI listed insect growth regulator insecticide with the active ingredient azadirachtin; 3) Ecotec (Brandt Consolidated, Inc.), an OMRI listed insecticide/miticide with a combination of essential oils as the active ingredients; 4) OxiDate 2.0 (BioSafe Systems LLC), an OMRI listed antimicrobial with the active ingredients hydrogen dioxide and peroxyacetic acid; 5) Agri-Mycin 17 (Nufarm Limited), an antimicrobial with the active ingredient streptomycin sulfate; and 6) Liquid Copper Fungicide (Southern Agricultural Insecticides, Inc.), an antimicrobial with the active ingredient copper diammonia diacetate complex.

Concentrations used were the label rate of each product for use in orchard crops (pome and stone fruits). For labels with directions for use by crop area, a diluted spray volume of 948 L per hectare, after consulting with research farm managers who stated that typically 477 to 948 L per hectare are used to achieve runoff in fruit orchards. Products were mixed with water in 100 mL aliquots according to the following concentrations: Naiad at 0.5% by volume (surfactants are typically used at 0.5% to 1% by volume); AzaGuard at .125% by volume (1.18 L per hectare); Ecotec at .5% by volume (4.74 L per hectare); OxiDate 2.0 at .25% by volume (1 : 400, volume : volume); Agri-Mycin at 100 ppm (60 mg per 100 mL); and Liquid Copper Fungicide at .78% by volume (29.6 ml per 3785 ml).

Egg mass treatments

A total of 119 egg masses were collected over the course of two months on nine collection dates. At each date, egg masses were randomly allocated to seven treatments (one control and six products). The number of egg masses receiving each treatment varied with collection date, but collectively totaled 17 egg masses per

treatment. Egg masses assigned to the control treatment were not manipulated in any way, whereas egg masses assigned to the products were treated with a diluted solution of each respective product. The treatment solutions were applied to the egg masses using a hobbyist paint sprayer (Master Airbrush Model Kit-G23-22), which produced a fine, even mist that served to mimic the small droplet size created by an airblast pesticide sprayer. In a fume hood, the paint sprayer was held at a 45 degree angle approximately 18 cm away from the leaf disk with the egg mass, which was secured with tweezers, and the sprayed solution was delivered for approximately five seconds in a sweeping hand motion until saturation or runoff was achieved. Between treatments, the sprayer was triple rinsed with clean water and sprayed clear for 10 seconds to remove any remaining residual product.

Nymphal rearing

After treatment, the number of eggs per mass was recorded, and the leaf disk with each egg mass was placed individually on a leaf terminal of a green bean plant, which was inserted into a 15 mL floral water pick and placed in a clear plastic deli container (16x14x5 cm) with a screened opening in the lid. For additional food, a green bean was placed in each container. Containers were held in an environmental chamber under favorable rearing conditions (25°C, 65-75% humidity, 16 hour L: D diurnal cycle). Leaf terminals and green beans were replaced as needed to maintain consistent freshness of the food. Dishes were misted with water and observed for hatching on a daily basis. Once hatch occurred, the number of hatched eggs was recorded, and nymphs were then observed daily. Dead nymphs were removed each day, and survival was recorded until molt to the second instar. As nymphs molted to

the second instar, they were killed in 100% ethanol and stored at -20° F until PCR analysis.

DNA extraction and PCR analysis

Each second instar used for PCR analysis was rinsed in an ethanol bath before being shredded with tweezers and transferred to a collection tube for DNA extraction using a Qiagen DNEasy Extraction Kit. Because of their small size, whole second instars were used instead of attempting to separate the gut contents. Due to time and budget restraints, it was not possible to screen all of the nymphs surviving from each egg mass in the study. Instead, a subset of 6 to 9 egg masses from the possible 17 masses per treatment was randomly selected. Of these egg masses, a random sample of 10 nymphs was analyzed individually by PCR to determine the percentage of nymphs inoculated with the symbiont bacteria, about 550 nymphs screened in total. Attempts were made to choose at least one egg mass from each of the nine collection dates in order to treat date as a random blocking factor; however, some egg masses did not hatch in a given collection date.

PCR and gel electrophoresis were used to determine the presence or absence of the symbiont which indicated whether the nymphs were able to successfully acquire the bacteria from the egg mass surface. Primers specific to *P. carbekii* were used to screen each nymph (Figure 4.1) (~930bp, forward: GCATATAAAGATTTTACTCTTTAGGTGGC and reverse: CTCGAAAGCACCAATCCATTTCT) (Bansal et al. 2014). For samples that did not amplify symbiont DNA, positive control primers for stink bug mitochondrial DNA were used to determine that the symbiont was truly absent from a sample and not missing due to a sample quality (Figure 4.2) (147bp, forward:

CGAATCCCATTGTTTGTGTG and reverse: AGGGTCTCCTCCTCCTGATG (Kumar and Dunning-Hotopp, unpublished data). Any indication of a distinct rectangular gel band, no matter how faint, was considered 'positive' for all samples.

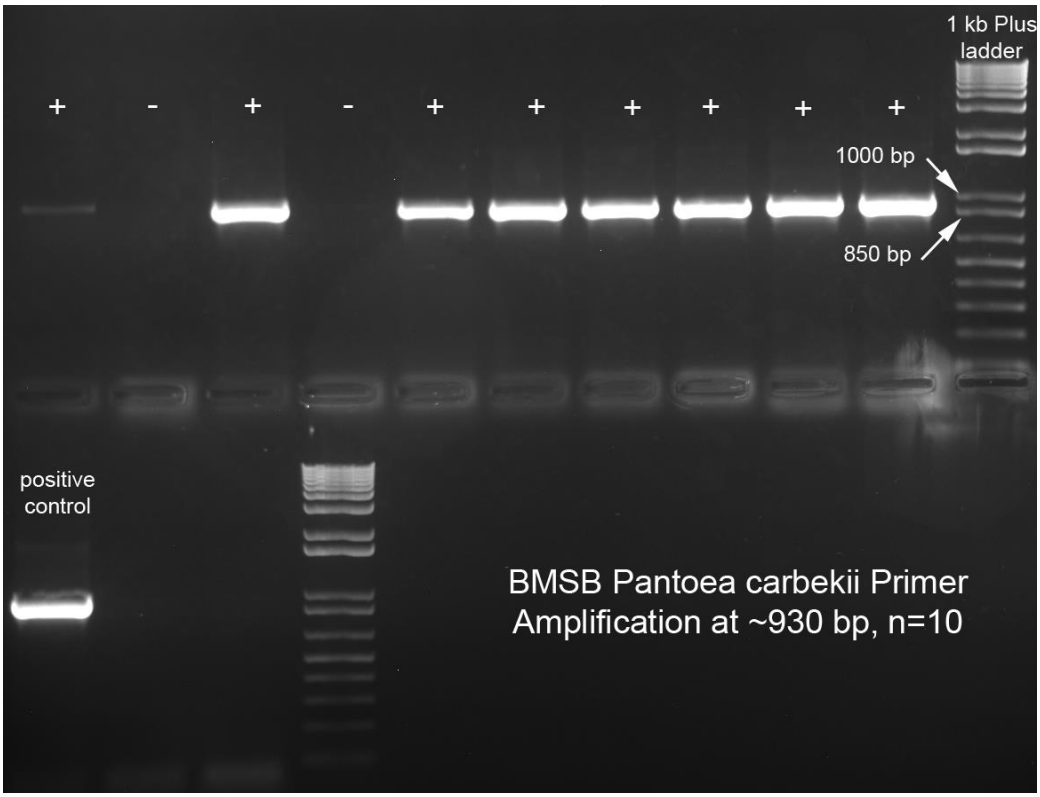

Figure 4.1. Example of gel electrophoresis with PCR products using *H. halys* second instar DNA and *P. carbekii* primers; 8 out of the 10 nymphs and the positive control sample tested positively for the symbiont in this screening of egg mass rep one from the Ecotec treatment, and gel band amplification occurred at the appropriate location on the ladder for all 9 positive samples (between the 850 and 1000bp ladder bands) with no amplification in the extraction blank or PCR blank. Note that there were differences in gel band intensity.

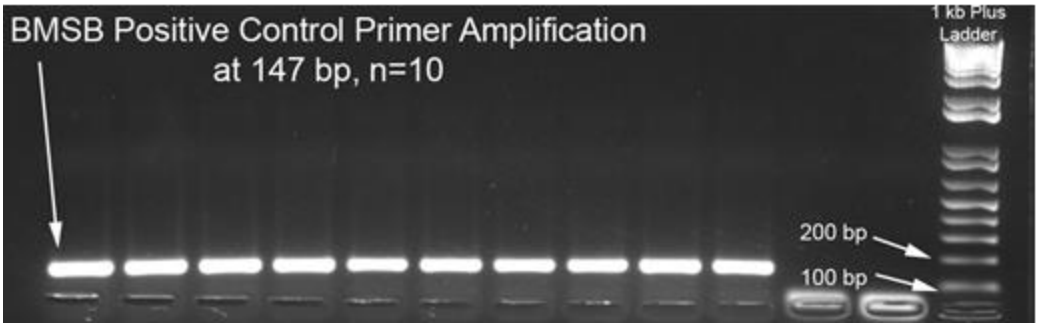

Figure 4.2. Example of gel electrophoresis with PCR products using *H. halys* second instar DNA and mitochondrial DNA primers; all 10 nymphs from egg mass rep 14 for the Naiad treatment in the sample tested negatively for the symbiont in a previous PCR reaction using symbiont primers, but those same DNA samples tested positively for stink bug mitochondrial DNA above, verifying sample quality. Gel band amplification for the mitochondrial DNA occurred at the appropriate location on the ladder (between the 100 and 200bp ladder bands) with no amplification in the extraction blank or PCR blank.

Data analysis

Data from the laboratory study were summarized as percent egg hatch, percent survival to the second instar, and percent of nymphs with symbiont inoculation. Each variable was analyzed as a one-way ANOVA using the Proc Mixed function in SAS to test for treatment effects. Because each egg mass was individually treated with a new product solution, egg mass was treated as the replicate unit. The collection date was considered a random blocking factor to remove possible variance due to conditions when the egg masses were collected from the colony.

Materials and Methods: Field Study

Insect culture

Due to low *H. halys* populations at the University of Maryland research farms during the late summer months of 2015, adults were collected from farm sites in Virginia. The stink bugs were reared in the laboratory under the same conditions as

described in the laboratory study. The egg masses used for this study were all collected on one day during peak egg production.

Product information

The field study tested four treatments, consisting of a water control, Naiad, AzaGuard, and Triton-X-100 (The Dow Chemical Company), a nonionic octylphenol ethoxylate surfactant. Naiad and AzaGuard were chosen based on their overall ability to sterilize egg masses in the laboratory study. Triton-X-100 was included because results from a preliminary trial with OxiDate 2.0 in combination with Triton-X-100 showed an increase in the ability to sterilize egg masses, whereas OxiDate 2.0 alone did not. Concentrations were as follows: Naiad at 1% by volume (2X the concentration by volume used in the laboratory study); Triton-X-100 at 1% by volume; and AzaGuard at 1.67 L per hectare (the high range of the recommended label rate).

Field location and experimental design

The field study was conducted at the University of Maryland Wye Research and Education facility (Queenstown, MD) in a peach orchard. Four rows of trees were selected within the orchard, each separated by at least one row of trees. In each row, four trees were selected with at least two trees between them to avoid spray drift exposure. Trees within each row was assigned different treatments in a Latin-square design for a total of 16 trees. Each tree was treated as a replicate unit.

Egg mass preparation and treatment application

A total of 36 egg masses on leaf disks were removed from the rearing colony on one day during peak egg production. Double sided photo album tape was used to secure each egg mass to a 5 x 5 cm index card cutout. Sand was poured over the

exposed adhesive surface of the tape, so that nymphs would not become stuck upon hatching. Owing to a limited supply of egg masses from the colony, groups of two egg masses were randomly allocated to three replicate trees and three egg masses to one replicate tree for each treatment. Egg masses were attached with a staple to the underside of randomly chosen leaves in the outer canopy on one side of the respective tree, about 1.5 m above the ground at eye level. Egg masses within a tree were treated as subsamples.

Product mixtures were applied using a backpack mist sprayer (Solo, Newport News, VA), set to deliver a spray volume of 3.44 L per minute. Given this output rate at full throttle, the spray volume per hectare was estimated by measuring the amount of water required to spray several trees until runoff. The spray volume was 630 L per hectare based on an average of 1.7 L per tree, each occupying 27 m^2 of a hectare. This amount represented approximately the mid-range spray volume used in tree fruit applications with a commercial airblast sprayer. Once calibrated, each product was mixed in the holding tank of the sprayer with 11.4 L of water at the appropriate concentration and applied to runoff to one side of each replicate tree assigned to that treatment. Treatments were applied one product at a time to all four trees. The holding tank was triple rinsed between treatments. Egg masses were collected approximately 30 minutes later, once the foliage had dried, and brought back to the laboratory.

Nymphal rearing and PCR analysis

Egg masses were reared under the same conditions as described in the laboratory study, except that the two or three egg masses per replicate were reared together in one deli container. The number of hatched eggs was recorded for each egg

mass and then averaged over all masses per replicate. The newly hatched nymphs in each container were reared to determine the number surviving to the second instar. As nymphs molted to the second instar, they were killed in 100% ethanol and stored at -20° F until PCR analysis. Random samples of five nymphs per replicate per treatment were selected for PCR analysis to determine presence or absence of the symbiont. DNA extraction and PCR was conducted according to the same methods described in the laboratory study.

Data analysis

Data analysis was conducted using the Proc Mixed function in SAS to test for treatment differences in the percentage of hatched eggs, survival to the second instar, and percent symbiont inoculation. Row and column tree positions were used as blocking factors but the mixed model removed these random factors if they did not significantly contribute to the total variance.

Results: Laboratory Study

There was a significant treatment effect on the percentage of hatched eggs ($F_{(6,105)} = 5.56$, $p<0.001$), survival of nymphs to the second instar ($F_{(6,100)} = 4.4$, $p<0.001$), and percentage of second instars inoculated with the symbiont bacteria ($F_{(6,41.5)} = 7.3$, $p<0.0001$). Average hatch rate (± SE) of the control treatment was considered normal at 91.3% ± 3.7, and not significantly different from the Ecotec treatment (90.8% ± 2.3), AzaGuard (84.7% ± 3.6), OxiDate 2.0 (88.7% ± 4.6), or Liquid Copper Fungicide (92.3% ± 1.9) (Fig. 4.3). However, the surfactant Naiad (60.0% ± 9.2) and Agri-Mycin (74.4% ± 8.2) significantly reduced the rate of egg hatch by 18.5 to 34.2%. Average percent survival (± SE) to the second instar was

significantly reduced by the Naiad treatment (65.9% ± 9.0) compared to the control survival at 82.0% ± 4.7 (Fig. 4.4). Although AzaGuard had no effect on egg hatch, this insect growth regulator significantly reduced nymphal survival (63.0% ± 5.9). Survival of nymphs from the Agri-Mycin treated egg masses was numerically lower (88.0% ± 3.0) but not significantly different from that of the control treatment. Treatments of Ecotec (87.0% ± 3.5), OxiDate 2.0 (85.3% ± 3.8), and Liquid Copper Fungicide (78.1% ± 4.0) (Figure 4.4) had no effect on nymphal survival.

Most (89% ± 4.5 SE) of second instars surviving from untreated egg masses were inoculated with the symbiont bacteria (Figure 4.5). Although numerically lower, the inoculation rates of second instars from egg masses treated with Ecotec (67.5% ± 12.5), Agri-Mycin (75.7% ±6.5), and OxiDate 2.0 (73.7% ± 12.1) were not significant different from that of the control treatment. Egg mass treatments of Naiad (23.6% ± 12.3), AzaGuard (24.1% ± 9.5), and Liquid Copper Fungicide (35.6% ± 11.0) significantly reduced the percentage of inoculated second instars by as much as 73.5% compared to the control treatment.

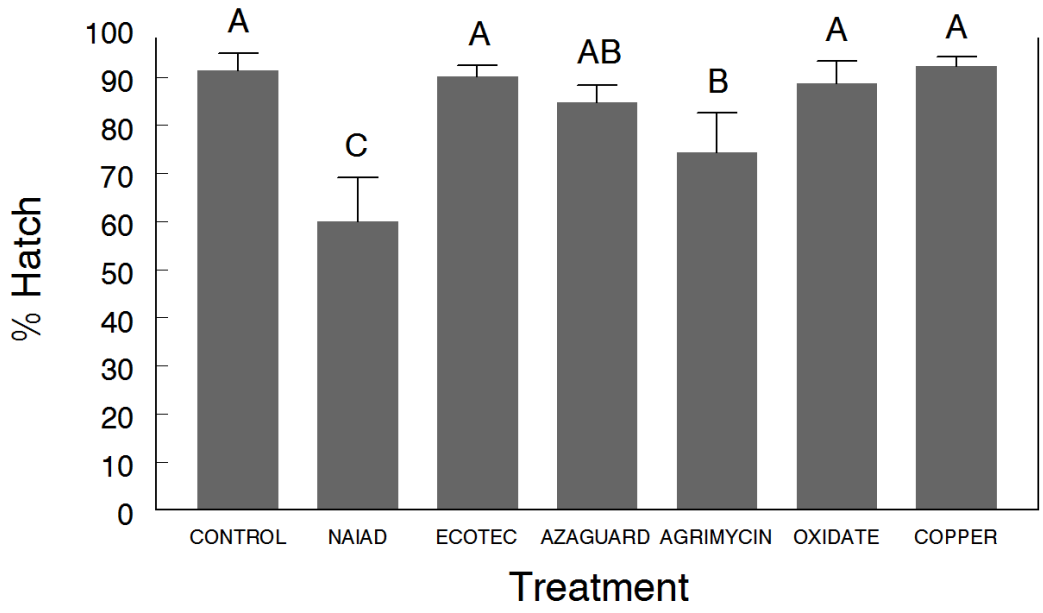

Figure 4.3. Effects on the percentage of *H. halys* eggs that hatched by selected antimicrobials and surfactants in water solutions sprayed on egg masses in the laboratory. Comparisons of means (± SE) with the same letter are not significantly different (*p=0.05*). Means for each treatment are based on 17 replicate egg masses.

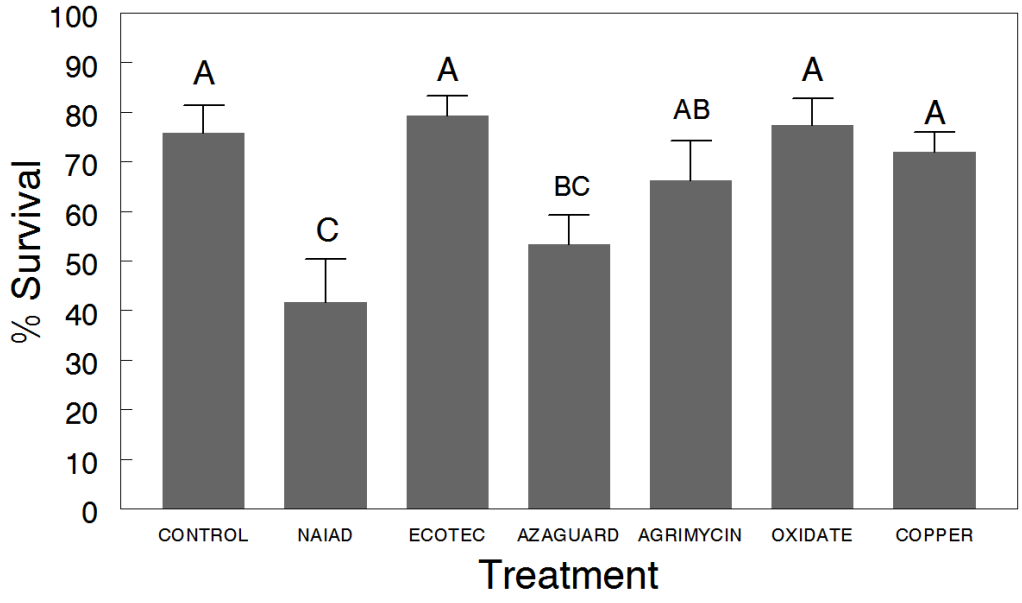

Figure 4.4. Effects on the percentage of *H. halys* nymphs that survived to the second instar by selected antimicrobials and surfactants in water solutions sprayed on egg masses in the laboratory. Comparisons of means (± SE) with the same letter are not significantly different (*p=0.05*). Means for each treatment are based on 17 replicate egg masses.

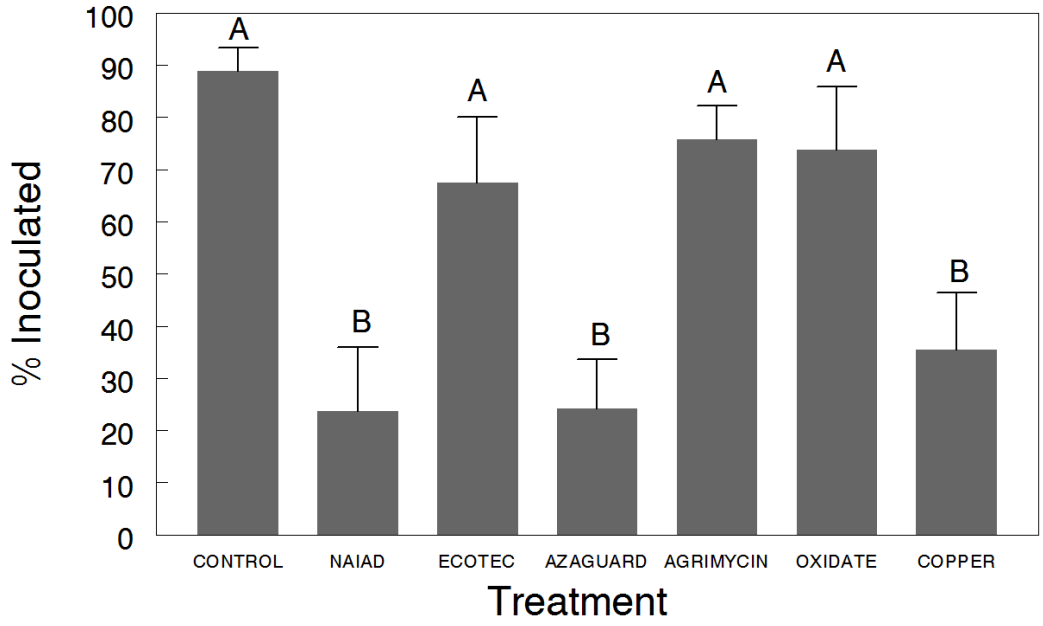

Figure 4.5. Effects on the percentage of *H. halys* nymphs that successfully acquired their symbiont by selected antimicrobials and surfactants in water solutions sprayed on egg masses in the laboratory. Comparisons of means (± SE) with the same letter are not significantly different (*p=0.05*). Means for each treatment are based on either six (AzaGuard), seven (Agri-Mycin 17), eight (Naiad, Ecotec, OxiDate 2.0) or nine (Control, Liquid Copper Fungicide) replicate egg masses

Results: Field Study

There was a significant treatment effect on egg hatch rate ($F_{(3,12)} = 5.32$, p= <0.015), and both surfactants, Naiad and Triton-X-100, reduced the number of eggs hatching by 10 to 16% compared to the hatch rate of untreated egg masses (94.5% ± 2.7 SE) (Figure 4.6A). The hatch rate of egg masses on AzaGuard-treated trees was not affected. Although there were lower survival rates of second instars from egg masses on trees treated with Naiad and Triton-X (Figure 4.6B), nymphal survival, including the AzaGuard treatment, were not statistically different from that of the untreated egg masses ($F_{(3,12)}$= 1.1, p= 0.39). Similarly, none of the treatments had any significant impact on acquirement of symbiont bacteria from the surface of the egg masses ($F_{(3,6)}$= 0.2, p= 0.89) (Figure 4.6C).

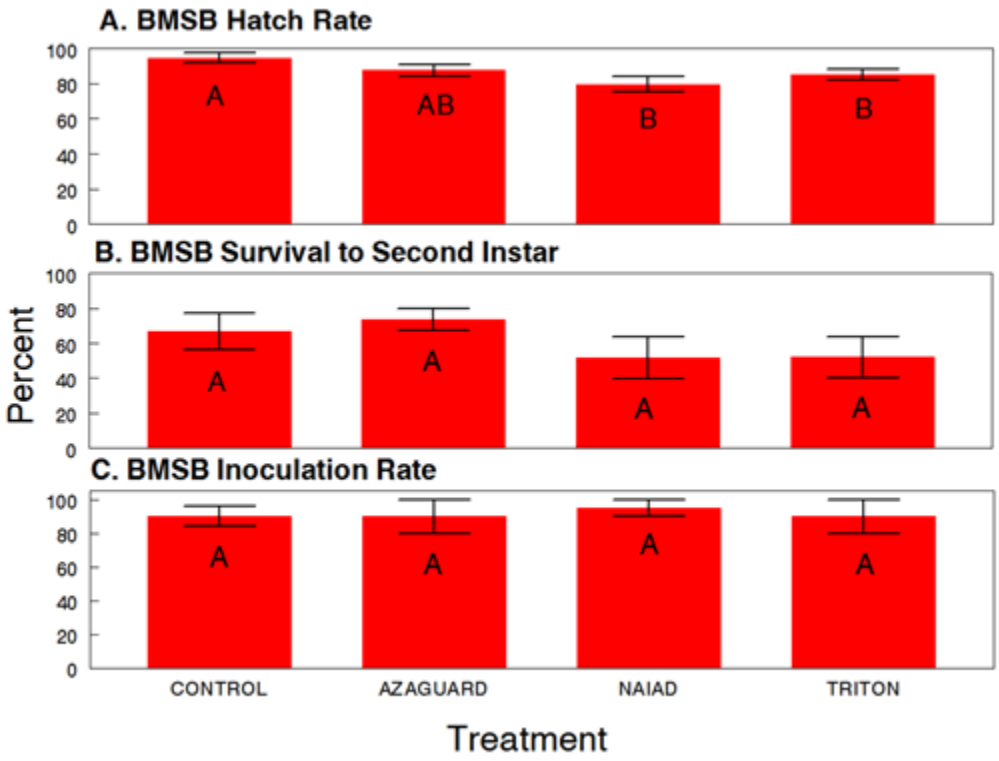

Figure 4.6A-C. Effects on the percentage of nymphs that (A) hatched, (B) survived to the second instar, and (C) successfully acquired their symbiont, by selected antimicrobials and surfactants in water solutions sprayed on egg masses in the field with a backpack airblast sprayer. Comparisons of means (± SE) with the same letter are not significantly different ($p=0.05$). Means for each treatment are based on four replicate trees, each with between two to three egg mass sub samples per tree.

Discussion

Like some other species of stink bugs, *H. halys* relies on the presence of another organism, the bacterial symbiont *P. carbekii*, for successful survival and development. Loss of the symbiont has devastating effects on the well-being of *H. halys*, and can be viewed as a weak link in its life history. The behavior of smearing the symbiont on the egg mass leaves the symbiont exposed on the egg mass surface until the nymphs hatch and acquire it. This study is the first to evaluate the effects of antimicrobial and surfactant applications on egg hatchability, the fitness of *H. halys* nymphs, and successful symbiont acquisition. Taken altogether, laboratory and field

results support the hypothesis that egg mass exposure to these products can cause direct effects on *H. halys* egg hatch rate and subsequent nymphal survival, as well as lessen the success that newly hatched nymphs have at obtaining the necessary symbiont from the egg mass surface. In the laboratory study, hatch rate was significantly reduced by two of the six treatments (Agri-Mycin and Naiad), survival was significantly impacted by two of the six treatments (AzaGuard and Naiad), and symbiont acquisition success was disrupted by three of the six treatments (Naiad, AzaGuard and Liquid Copper Fungicide). Under field conditions, there were no significant differences among treatments on nymphal survival or symbiont acquisition; however, significant reductions in average hatch rate were detected for the two surfactant treatments (Naiad and Triton-X) when compared to that of the control egg masses.

Kenyon et al. (2015) reported that the symbiotic bacteria required by *H. halys* are indeed located on the egg mass surface, but also that the eggs are covered in a secretion, with the bacteria located underneath and intercalated within this secretion. The fact that these gut inhabiting bacteria are not merely exposed on the surface of the egg mass is probably the reason they are able to survive on the egg mass surface for about a week until nymphs hatch and acquire them. The secreted coating that apparently protects the bacteria also makes it difficult for any chemical application to impact the bacteria and manage *H. halys*. Although egg masses were sufficiently exposed to treatments in the laboratory study, several antimicrobial products tested were ineffective at preventing symbiont acquisition, and this was most likely due to the inability to penetrate the extracellular coating to contact the bacteria. It is

unknown how newly-hatched nymphs bypass this coating to inoculate themselves, but they likely regurgitate saliva to break down the coating in order to imbibe it along with the symbiont. In these studies some products adversely impacted survival of the second instar, indicating that the nymphs were orally exposed to treatment residues on the egg mass surface while inoculating themselves. This occurred in the AzaGuard treatment, which requires ingestion to be effective.

In the laboratory study, surfactants were most effective. They are the group of products to explore in future work, owing to their ability to remove or penetrate the secreted coating. Egg mass treatments of the surfactant Naiad not only significantly impacted hatch rate and survival to the second instar, but also reduced symbiont acquisition. Not surprisingly, insect growth regulator AzaGuard significantly impacted survival to the second instar but also significantly reduced symbiont acquisition. It should be noted that this product is formulated with surfactants, which might explain in part its ability to successfully kill the bacteria. Surfactants have also been shown to work as antimicrobials for plant diseases (Stanghellini 1987, 1996, Stanghellini and Miller 1997, Irish et al. 2002, Mickler 2002) by disrupting certain cell membranes of fungal structures. Products with both antimicrobial effects and the ability to penetrate the coating covering the bacteria provide the best chance for disrupting symbiont acquisition.

In contrast to the laboratory study, there were fewer treatment effects when products were applied to egg masses on peach trees to simulate a commercial airblast application. The two surfactants did affect egg hatch to a small but significant degree but had no detectable effects on nymphal survival or symbiont acquisition for several

possible reasons. First, although the treatment solutions were sprayed on trees to run off, actual exposure to the attached egg masses may not have been sufficient to affect the egg mass coating and the bacteria underneath. Secondly, the supply of colony-reared egg masses was unfortunately limited at the time of the field study, and there was some evidence of disease infection in the laboratory colony which could have affected nymphal fitness. Additionally, given the high variance in the data, the level of replication and precision in the field study was not adequate to detect statistical differences. Nevertheless, the combined results support the hypothesis that antimicrobials and surfactants can adversely affect *H. halys* egg and nymphal fitness and disrupt symbiont acquisition and thus have the potential to be used as a management tool. However, further research is needed to more rigorously evaluate different treatments applied under field conditions, particularly possible synergistic combinations of antimicrobials and surfactants to improve the ability of a treatment to penetrate the egg mass coating and sterilize the bacteria.

Acknowledgements

I thank Dr. Kathryne Everts for her assistance in choosing the products tested in the study, as well as Dr. Julie Dunning-Hotopp and Nikhil Kumar for providing the positive control primers used to test the quality of the DNA samples. I also thank Dr. Thomas Kuhar for providing locations in Virginia to collect *H. halys* adults when populations were low in Maryland.

Conclusions and implications

One of the most important steps in developing management strategies for an insect pest is to develop a thorough understanding of the biology of that pest. The brown marmorated stink bug, *H. halys*, a native species in Southeast Asia, was accidentally introduced into the United States in the late 1990's, and since that introduction it has established itself as a major nuisance and economic problem here as an invasive species. My dissertation sought to develop insight into an important aspect of the biology of *H. halys*, its relationship with bacterial symbionts.

Research into this particular aspect of the bug's life history was necessary because symbiotic relationships in many other insects are crucial to the successful survival of their respective insect hosts. My research confirmed that the brown marmorated stink bug, like many other species of insects within the order Hemiptera, has developed a close knit relationship with an obligate symbiont, *P. carbekii*, which colonizes the crypts of the fourth region of the midgut as well as the surface of the egg mass when it is laid. My work confirmed that the absence of this symbiont has devastating impacts on the fitness of *H. halys*, negatively affecting survival, development, fecundity, and even resulting in aberrant behavior in the first instar. However, there are no hard and fast rules when it comes to level of symbiont dependence across different groups of insects, or even within families such as the Pentatomidae. Studies into these microbial associations are clearly an important step if we are to understand the biology of future invasive species, and should be considered a priority.

Because *H. halys* is so reliant on *P. carbekii*, it is reasonable to assume that when the symbiont suffers, so does its host. One stressor that is commonly documented in the literature is temperature, which can impact both insects and symbionts. There are countless studies that have documented fitness reductions in insects at higher temperatures, and for insects that harbor symbionts, there are studies that document symbiont loss at sometimes even minor up shifts in temperature (such as aphids and stink bugs). One review in particular by Wernegreen (2012) suggests that symbionts put constraints on their hosts and their range expanding capabilities due to their intolerance to heat stress. Although many symbionts, *P. carbekii* included, have stress response genes present, they still show high mortality when exposed to higher temperatures. Wilcox et al. 2003 showed that the aphid symbiont *Buchnera aphidicola* does have stress response genes present, but only a quarter of them are differentially expressed under temperature changes, and those that do only have very modest changes in expression. In addition, those stress response genes are constantly highly expressed even under normal conditions. Symbionts are very prone to physiological issues due to their reduced genome, and one of the biggest problems that they face is protein misfolding and degradation. The enzymes produced by these stress response genes can actually rescue misfolded proteins to a degree, and studies have shown that this included rescuing misfolded proteins caused by mutations. Therefore it is likely that the presence of those stress response genes is more of a way to deal with the physiological problems that the symbiont faces and not to deal with fluctuating environmental conditions. My studies revealed a decrease in survival, longer development, and an almost complete halt in egg mass production when *H.*

halys was exposed to a temperature increase from 25 to 30 degrees C, and analysis of adults revealed a lower percentage of adults tested positively for the symbiont at the higher temperature. It is interesting to note that Venugopal et al. (2016) reported that temperature is the most important factor in determining *H. halys* populations in the Mid Atlantic, and that the hotter it is, the fewer stink bugs there will be. The range expanding capabilities of *H. halys* may indeed be largely driven by the tolerances of its symbiont, and may explain patterns of species distribution for other insects that harbor symbionts. More research into the impacts of real world temperature conditions on the outskirts of the range of *H. halys* in the United State may reveal the symbiont as a major player in dictating where *H. halys* can actually thrive.

One of the easiest factors to manipulate during my dissertation studies was the symbiont itself, due to its presence on the egg mass surface. My sterilization protocols inspired the idea that the symbiont was in fact targetable on the egg mass as a possible management technique, if you think of it as a bacterial 'infection' that you want to eliminate. The screening of a variety of commercial products revealed that not only is the symbiont vulnerable, but so is the host insect. Direct effects on nymphal survival when egg masses were treated with the insect growth regulator azadirachtin revealed that the probing behavior of the first instar nymphs makes them vulnerable to the ingestion of residues on their egg mass. Future work into egg mass treatments may reveal a useful and novel way to expose newly hatched stink bugs to insecticides. However, my findings also demonstrated that the symbiont can be sterilized from the egg mass surface, although this is not as easily accomplished as it was initially believed to be. The presence of the egg mass coating proved to be a

major hurdle in implementing this strategy, since only surfactants or products with surfactants as inert ingredients seemed to be able to penetrate the coating and eliminate the bacteria underneath. Future work that looks into the composition of this egg mass coating may provide insight into other types of products necessary to break it down. My ability to test this strategy in the field was limited, but future work into the best strategies for optimizing coverage seems to be the logical next step. The possible synergistic effects of antimicrobials in combination with surfactants may reveal an even more successful capability of egg mass sterilization.

In conclusion, my dissertation explored another important insect- microbe association, and provides some evidence to suggest that the symbiont is playing a large role in driving the range expanding capabilities of *H. halys*. Additionally, I explored a novel way to manage an insect pest by targeting its symbiont, and described some of the hurdles and future work needed to explore this potential management strategy.

Appendix A: Approval for previously published work

To the Dean of the Graduate School:

Christopher Michael Taylor, a Ph.D. candidate within the Department of Entomology, wishes to include the following publication as the second chapter of his dissertation, 'Understanding the Relationship between the Brown Marmorated Stink Bug, *Halyomorpha halys* (Stål), and its symbiont, *Candidatus Pantoea carbekii*, with implications for stink bug management':

> Taylor CM, Coffey PL, DeLay BD, Dively GP (2014) The Importance of Gut Symbionts in the Development of the Brown Marmorated Stink Bug, *Halyomorpha halys* (Stål). PLoS ONE 9(3): e90312. doi:10.1371/journal.pone.0090312.

We, the examining committee of Christopher Michael Taylor, have determined that Christopher made a substantial contribution to the aforementioned publication. The inclusion of this work in his dissertation has the approval of his dissertation committee, dissertation adviser Dr. Charles Mitter, and the Graduate Director of the Department of Entomology Dr. Jeffrey Schultz.

Signature _____ Date: 4/11/16 Graduate Director

Signature _____ Date: 4/12/2016 Adviser

Signature _____ Date: 4/12/2016 Committee Member

Signature _____ Date: 4/12/16 Committee Member

Signature _____ Date: 4/12/16 Committee Member

Signature _____ Date: 04/12/16 Committee Member

Signature _____ Date: 4-12-16 Committee Member

Appendix B: Commentary on determining the optimal egg mass soaking time to generate sterilized egg masses

Kenyon et al. (2015) determined that the *H. halys* egg mass is covered in a secretion by the female either during or after the egg mass laying process, and that the symbiont *P. carbekii* is located underneath and intercalated within this secretion. Interestingly enough, it also appears that this coating actually holds the egg mass cluster together, since removal of this coating makes the eggs within the egg mass separate from each other. Prado et al. (2006) and Prado and Almeida (2009a) made note of this in their studies with *Nezara viridula*, *Murgantia histrionica*, and *Chinavia hilaris*, and suggested that optimizing the soaking time should be done for each species of stink bug.

For *H. halys*, I followed a modified soaking protocol from that seen in Prado et al. (2006) and Prado and Almeida (2009a). Egg masses were first soaked in a 100% ethanol bath for 5 minutes, then submerged in a 10% Clorox solution for 6 minutes, and then rinsed in a separate 100% ethanol bath followed by a distilled water bath. I determined how long to soak the egg masses in the 10% Clorox bleach solution by conducting a side study where I soaked egg masses for either 2, 4, 6, or 8 minutes, and recording what percentage of the egg masses fell apart for each soaking time. There was not much of a difference at 2, 4, and 6 minutes, but most of the egg masses soaking for 8 minutes fell apart, so we opted to go with a 6 minute bleach soak so that they were sterilized for as long as possible.

I will note that we left egg masses on the leaf disks that we cut out of the leaves they were laid on with a cork borer, which served as a surface to help hold the

egg masses together when they started to dissociate. Egg masses removed from the mesh screen very rarely stayed together at any soak time, so it is important to leave the egg mass on the substrate on which it was laid.

Because you cannot completely remove the coating without the egg mass falling apart, some symbiont might be left on the egg mass and sterilization is not 100% complete. In my studies I did have a few stink bugs test positively for the symbiont from the sterilized egg mass treatments, although the percent that tested positively was always much lower than the control stink bug symbiont presence. This is the unfortunate but necessary tradeoff to avoid the risk of negative effects on stink bug fitness when the egg mass completely falls apart. Further studies into optimizing this technique even further would be valuable.

Appendix C: Commentary on observing the first instar egg mass chorion probing behavior

Many research papers state that, for the family Pentatomidae, the obligate symbiont is usually deposited on the egg mass surface and that the newly hatched nymphs must probe the egg mass to inoculate themselves with the symbiont. Although this fact has been stated and cited many times, no description of any behavioral observations has been published. Here I describe my confirmation of this post hatch behavior for *H. halys* from my observations during my dissertation work.

In the early stages of my research, attempts to observe probing behavior were not successful. Nymphs (by the time they were fully sclerotized and exhibiting their typical red and black color pattern) that were clustered around the egg mass did not demonstrate any type of behavior that would indicate an attempt to acquire their symbiont. It wasn't until I conducted a large scale study with many egg masses that I was able to confirm symbiont acquisition behavior. Due to the nature of that particular study, I was checking egg masses constantly, and would sometimes be able to observe an egg mass the moment it was hatching. Nymphs hatch synchronously, and as soon as they exited their eggs, they were observed very actively roaming the egg mass chorions. Closer examination through a microscope confirmed that their mouthparts were extended and being raked over and poked in between the egg mass chorions as they moved about.

After a few minutes of active probing, a nymph would cease movement, and settle on a particular spot. Close observation of the head capsule revealed that the head capsule would 'collapse' for a moment before popping back into its original

position, confirming sucking behavior. Whether or not the nymph regurgitates saliva or some other material to imbibe the symbiont was not confirmed, but due to the presence of an egg mass coating that covers the symbiont (Kenyon et al. 2015), some sort of digestive secretion is likely necessary to break down that coating to expose the symbiont for nymphal acquisition. What composes this egg mass coating has not been described, but since it is likely imbibed along with the symbiont, it may have some sort of nutritional benefit for the newly hatched nymphs. First instar nymphs in the Pentatomidae typically don't begin feeding until days after hatch.

This egg mass roaming activity and subsequent sucking behavior was only observed for a few hours at most after hatch, demonstrating that symbiont acquisition is an immediate post hatch behavior, at least for *H. halys*. It would be interesting to see what the timeframe for symbiont acquisition is in other stink bug species.

Appendix D: Full protocol for DNA extraction, PCR analysis, and gel electrophoresis

Protocols for extraction, PCR, and gel electrophoresis were initially developed by Bridget DeLay during her time developing the *Pantoea* primers in 2013. I used these same protocols and altered them slightly when I switched to using the *P. carbekii* primers when they became available in 2014.

For DNA extraction a Qiagen DNEasy Blood and Tissue Kit was used in all studies. However, the initial sample preparation protocol differed slightly depending on whether adults or young instars were used. For adults, only the abdomen (along with all of its gut contents) was removed and used for extraction of DNA. When stink bugs had been stored for prolonged periods of time, it was difficult to find and remove just the fourth region of the midgut, so I opted to use the whole abdomen and its contents instead. For young instars, the entire insect was used for DNA extraction due to their small size. Regardless, in both cases the sample was well mashed in the centrifuge tube before lysis incubation. An extraction blank with no DNA was used during every round of DNA extraction to test for any contamination during the extraction process.

2x PCR PreMix with dye was bought from Syd Labs, Inc. and used for PCR reactions. The PreMix contained everything needed for a PCR reaction except the DNA template and primers. Each DNA sample was set up as a 20 microliter reaction, and contained the following:

1. 10 microliters 2x PCR PreMix with dye

2. 1.5 microliters forward *P. carbekii* primers (GCATATAAAGATTTTACTCTTTAGGTGGC, Bansal et al. 2014)
3. 1.5 microliters reverse *P. carbekii* primers (CTCGAAAGCACCAATCCATTTCT, Bansal et al. 2014)
4. 1 microliter DNA template
5. 6 microliters water

A PCR blank was set up with every round of PCR to test for any contamination during the PCR set up process. Once samples were set up, the PCR machine was programmed as follows:

1. Initial denaturation at 94° C for three minutes, then:
2. 35 cycles of 94° C for one minute, followed by 58° C for one minute, followed by72° C for two minutes, then:
3. Extension at 72° C for seven minutes, then:
4. Infinite run at 4° C so that reactions could run overnight and product could be removed from the machine at any time

PCR products were visualized using gel electrophoresis. A 1% agarose gel was made by adding 1.2 grams of agarose to 80 milliliters 1x TBE in a beaker. This was microwaved until the agarose had completely dissolved. Once it had cooled slightly, it was moved to the fume hood where 50 microliters of 0.6X ethidium bromide was added to the agarose solution. The beaker was swirled well to ensure even mixing. The agarose solution was then added to the gel rig, the gel combs were added, and the gel was allowed to cool until solid in the fume hood, about 30 minutes. Once cooled, loading buffer was added to the rig until the gel was covered. 8

microliters of PCR product (with dye already in the product) was pipetted into each well. 2 microliters of dye were combined with 2 microliters of 1 kb Plus DNA ladder (Thermo Fisher Scientific) and pipetted into the far right well of each lane. The gel was run at 105 volts for about 30 minutes, after which the gel was visualized using a UV camera and a picture was taken for my records and for future reference.

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