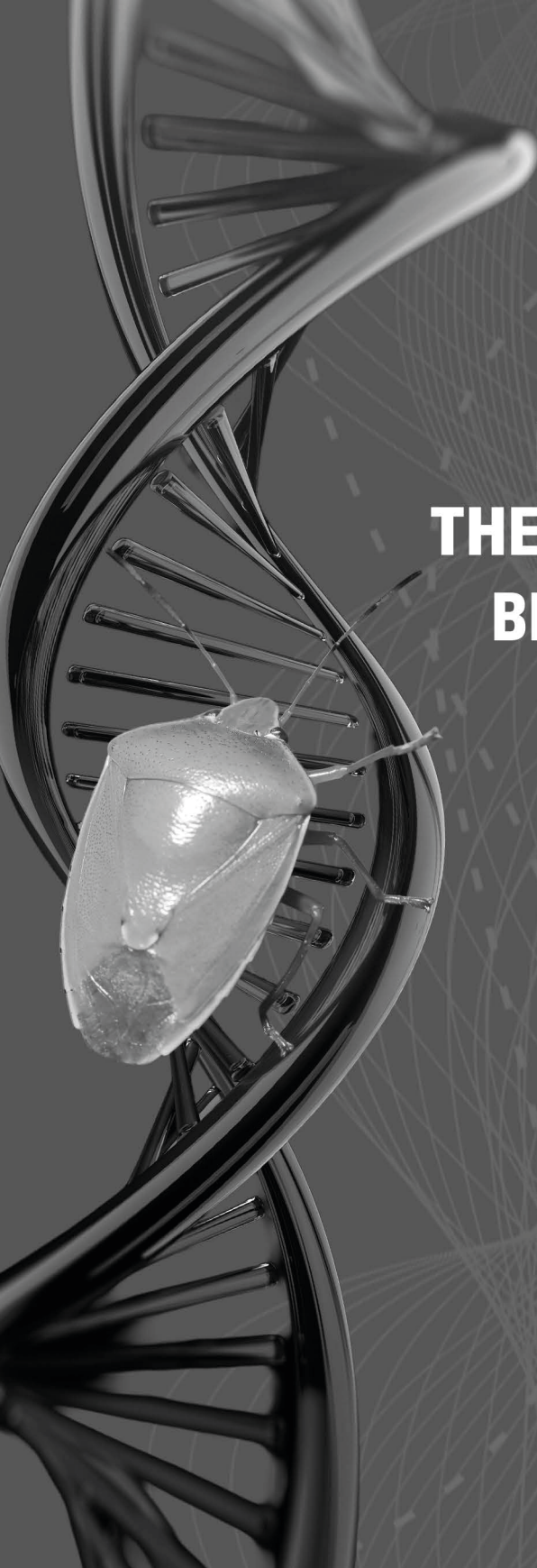
FOUNDATIONS OF THE MOLECULAR AND BIOLOGICAL STUDY OF SYMBIONTS IN PENTATOMIDAE (HEMIPTERA: HETEROPTERA)

Simone S. Prado

Atena Editora
Year 2026



FOUNDATIONS OF THE MOLECULAR AND BIOLOGICAL STUDY OF SYMBIONTS IN PENTATOMIDAE (HEMIPTERA: HETEROPTERA)

Simone S. Prado

Atena Editora
Year 2026

2025 by Atena Editora

Editor-in-chief
Prof. Dr. Antonella Carvalho de Oliveira
Executive Editor
Natalia Oliveira Scheffer
Images
iStock
Art editor
Yago Raphael Massuqueto Rocha

Atena Publishing is firmly committed to transparency and quality throughout the entire publication process. We work to ensure that everything is carried out ethically, avoiding issues such as plagiarism, data manipulation, or any external interference that could compromise the work.

If any suspicion of irregularity arises, it will be carefully reviewed and handled responsibly.

The content of the book, texts, data, and information, is the full responsibility of the author and does not necessarily represent the opinion of Atena Publishing. The work may be downloaded, shared, adapted, or freely reused, provided that both the author and the publisher are credited, in accordance with the Creative Commons Attribution 4.0 International License (CC BY 4.0).

Each work received careful attention from specialists before publication.

The Atena editorial team evaluated the national submissions, while external reviewers analyzed the materials from international authors.

All texts were approved based on criteria of impartiality and responsibility.

Foundations of the Molecular and Biological Study of Symbionts in Pentatomidae

| Organizer:
Simone de Souza Prado

| Revision:
The Authors

| Layout:
Nataly Gayde

| Cover:
Yago Raphael Massuqueto Rocha

Datos de catalogación en publicación internacional (CIP)

B615 Foundations of molecular and biological study of symbionts in pentatomidae (Hemiptera: Heteroptera) / Organizador Simone S. Prado. – Ponta Grossa - PR: Atena, 2026.

Formato: PDF
Requisitos del sistema: Adobe Acrobat Reader
Modo de acceso: World Wide Web
Incluye bibliografía
ISBN 978-65-258-3943-1
DOI: https://doi.org/10.22533/at.ed.431261601

1. Heterópteros - chinches verdaderas. I. Prado, Simone S. (Organizador). II. Título.
CDD 595.754

Preparado por Bibliotecario Janaina Ramos – CRB-8/9166

Atena Editora
+55 (42) 3323-5493
+55 (42) 99955-2866
www.atenaeditora.com.br
contato@atenaeditora.com.br

EDITORIAL BOARD

DEDICATION

To all my family members, especially to my wonderful parents Vitor Hugo Peetz Prado and Elza Maria de Souza Prado. Without their support, I would never have the courage to study abroad. To my siblings Vitor de Souza Prado and Silvia de Souza Prado, with whom I have shared all of my new discoveries while in USA. Finally, to my nephew Benicio Del Bianchi Prado and my goddaughter Júlia Aidar Marcondes, the youngest members of our family, who also contributed to the motivation behind the decision to publish this book.

ACKNOWLEDGMENTS

This book presents the research developed during my PhD and originally compiled from my doctoral thesis. This work was possible through the guidance and support of many colleagues and mentors in Hawaii and Berkeley. The chapters presented herein were developed as independent studies and were previously published individually in peer-reviewed scientific journals, in collaboration with numerous co-authors.

I would like to acknowledge those individuals that made this experience possible. First, I am eternally grateful to my advisor Rodrigo Almeida and my former advisor João Lopes for not only all their support and help, but also for providing encouragement with their contagious adventurous spirit.

I would also like to express my gratitude to all the faculty members and staff at UH Manoa, particularly to Mark Wright, Peter Follett, Dulal Borthakur, Dan Rubinoff, Ken Grace, Ron Mau, Julian Yates, Joseph Seifert, Anne Alvarez, Don McInnis and Mary Golden.

I would like to thank the all friends at UH, starting with the best lab mates that I could ever have: Jacqueline Robson and Mandy Anhalt. Second, Ranit Kirchbaumb, Darcy Oishi, Cerruti Hooks, Luc Leblanc, and Elsie Burbano, Leyla Valdivia, Adam Vorsino, Chris Kishimoto, Jesse Eiben, Cynthia King, Will Haines and Roshan Manandhar. I also those from UC Berkeley: Nick Hughes, John Chau, Nabil Killini, João Lopes, Mate Lopes, Chi-Wei Tsay, Fernanda Nascimento, Domenico Bosco, Matt Daugherty, Lisa Fernandez Kim Hung, Artem Ryazantsev, Leonora Bitleston, and Eileen Wong. To CNPq – Brazil for the financial support that allowed me to execute my project.

ABSTRACT

ABSTRACT

The Pentatomidae is one of the largest families within the suborder Heteroptera (order Hemiptera), with over 4000 described species within eight subfamilies. Stink bugs are major pests of economically important crops worldwide including soybeans, rice, pecan, cocoa and macadamia nuts. Relationships between microorganisms and insects have been previously studied, but the majority of these associations remain poorly understood. In this book, I present molecular data showing that *Nezara viridula, Acrosternum hilare, Murgantia histrionica, Euschistus heros*, *Chlorochroa ligata*, *Chlorochroa sayi*, *Chlorochroa uhleri*, *Plautia stali* and *Thyanta pallidovirens* harbor a common dominant caecum-associated bacterial symbiont in their midgut. I also show that oral rather than transovarial transmission occurs by detecting the symbiont on eggshells after nymphs had hatched, instead of detecting symbionts in female ovarioles. Additionally, I demonstrated that these symbionts are polyphyletic suggesting that replacement of the symbiont may occur over time. Using a demographic approach I analyzed the effect of egg masses surface sterilization and temperature on the symbiont maintenance and on insect development of the pentatomids *N. viridula*, *A. hilare* and *M. histrionica*. Here I demonstrate that maintenance of the symbiont was affected by high temperature and egg mass surface sterilization for the species *N. viridula*, *A. hilare* and *M. histrionica*. *N. viridula*'s reproduction parameters were not significantly different between the surface sterilized and control treatments at three temperatures, however no reproduction was observed at 20ºC of the surface sterilized treatment. Survivorship and reproductive parameters for *A. hilare* were negatively affected by surface sterilization of egg masses and for higher temperatures. Conversely, no effect was observed for *M. histrionica.* Here I suggest that the degree of mutualism of this association for pentatomid species is variable, given the fact that *A. hilare* requires its symbiont for adequate development, while *M. histrionica*'s symbiont may not be significant in development of the host insect and *N. viridula* is not affected by deprivation of its gut-associated symbiont.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Simone de Souza Prado

BACKGROUND

The Pentatomidae is one of the largest families within the suborder Heteroptera (order Hemiptera), with over 4000 described species within eight subfamilies (Panizzi et al. 2000). Many phytophagous pentatomid insects are polyphagous, feeding on a diverse range of plants. They are also major pests of economically important crops throughout the world including soybeans, rice, pecan, cocoa and macadamia nuts (Davis 1964, Panizzi et al. 2000). Stink bugs can cause direct and indirect damage by feeding on plant tissue with needle-like stylets and injecting digestive enzymes into plants tissue or providing free access to microbial infection, respectively (Jones and Caprio 1990, Panizzi et al. 2000). The economic importance of stink bugs is magnified as they are usually difficult to control (Daane et al. 2005).

Diverse insect groups that thrive on low nutrient diets, such as blood or plant sap, depend on obligatory mutualistic symbionts to provide additional nutrients required for the organism's survival (Douglas 1996). Relationships between microorganisms and insects have been previously studied, but the majority of these associations remain poorly understood. The best studied mutualistic bacterium-insect relationship occurs between the pea aphid *Acyrthosiphon pisum* (Hemiptera, Aphididae) and their primary symbiont *Buchnera aphidicola* (Shigenobu et al. 2000). Primary, or obligatory, endosymbionts are required for the survival of their insect hosts (Douglas 1996). *Buchnera aphidicola* has never been cultured outside its host and is present within specialized cells termed bacteriocytes (Buchner 1965, Shigenobu et al. 2000, Thao et al. 2002). Transovarial transmission is often the mode of symbiont transfer from one generation to another, a bottleneck that shapes the genome characteristics of the symbiont (Dale et al. 2002). Phylogenetic analyses have shown that, for certain insect families, insects and primary endosymbionts have coevolved

after a single initial infection for million of years (see Baumann, 2005 for review). Other examples of similar relationships are mealybugs and the symbiont *Tremblaya princeps*, whiteflies and *Portiera aleyrodidarum*, and sharpshooters and *Baumannia cicadellinicola* (Baumann 2005).

In contrast, secondary endosymbionts, also called facultative symbionts, are non-essential to their hosts, may be free-living and may not have specialized tissue localization (Dale and Maudlin 1999). These secondary symbionts may provide benefits to their hosts in the way of tolerance to heat stress, compensation for loss of primary symbionts, resistance to parasitic wasps, and to pathogens (Koga et al. 2003, Montllor et al. 2002, Oliver et al. 2005, Russell and Moran 2006, Scarbourough et al. 2005). Conversely, facultative symbionts can negatively impact the growth, reproduction, and longevity of their hosts (Chen et al. 2000). The evolutionary history of secondary endosymbionts often shows no coevolution with their hosts, suggesting multiple infections and/or horizontal transmission (Baumann 2005, Kikuchi et al. 2005). For example, *Sodalis glossinidius*, a secondary endosymbiont of tsetse flies can be cultured *in vitro* and apparently has not coevolved with the insect hosts (Dale et al. 2001, Matthew et al. 2005). *Sodalis glossinidius* is closely related to bacterial pathogens of insects, suggesting in this case that this symbiont evolved from an insect pathogen (Dale et al. 2001).

In the hemipteran suborder Heteroptera, Buchner (1965) discussed that endosymbionts are associated with the host's gut, and it has been suggested that symbiont transmission in some families occurs orally, not transovarially (Buchner 1965, Abe et al. 1995, Fukatsu and Hosokawa 2002). However, symbiont transmission strategies are not uniform for all families. Ingestion of excrement (coprophagy) is the mechanism used by *Rhodnius prolixus* (Stal) in the family Reduviidae (Beard et al. 2002); ingestion from a symbiont-filled capsule provided by the mother is the method used by *Megacopta punctatissima* (Montandon) in the family Plataspidae (Fukatsu and Hosokawa 2002, Hosokawa et al. 2005). In the family Alydidae, the species *Riptortus clavatus* (Thunberg) and *Leptocorisa chinensis* (Dallas) acquire the symbionts from the environment each generation (Kikuchi et al. 2005, 2007). For insects of the family Pentatomidae, it is been hypothesized that nymphs probe on the surface of eggs to acquire symbionts smeared onto the egg surface by the female, while ovipositing (Abe et al. 1995, Buchner 1965). Although not well documented, this unique transmission model, in which the symbionts live for a certain period of time outside the host, is interesting because it may allow for environmental factors to interfere with the symbiosis, and influence the coevolutionary interaction of hosts and symbionts.

Several biotic and abiotic factors affect insect's fitness and its relationship with symbionts. For example, high temperature may increase the developmental rate of organisms, including stink bugs (Vivan and Panizzi 2005). It has also been shown

that aphids submitted to high temperature treatments are negatively impacted due to the elimination of their primary symbionts (Ohtaka and Ishikawa 1991, Chen et al. 2000).

Demographic studies are an important tool used in the field of entomology. For stink bugs, several studies have employed demographical analyses to compare different rearing techniques and diets, to quantify life history traits at different temperatures and to compare efficiency of insecticides (Fortes et al. 2006, Legaspi 2004, Zanuncio et al. 2005). The role of bacterial symbionts in insect host populations has not been examined using demographic approaches. In this study, I used demographic approaches to study the effect of Pentatomidae gut symbionts on the fitness of the stink bugs.

In summary, I showed that insects of the family Pentatomidae harbor a dominant caecum-associated bacterial symbiont in their midgut. I also demonstrated that the pentatomid gut-associated symbionts are polyphyletic suggesting that replacement of the symbiont may occur over time. Phylogenetic placement showed the pentatomid symbionts were associated with plant pathogens. I analyzed the effect of egg masses surface sterilization and temperature on the symbiont maintenance and on insect development of the pentatomids *N. viridula*, *A. hilare* and *M. histrionica*, showing that the degree of mutualism in this system is variable. Overall results of this book enhanced our understanding of caeca-associated symbionts in pentatomid insects. The data acquired by the characterization of this symbiotic system will contribute to the understanding of pentatomid biology and ecology. In addition, this research provides information that may become important in the development of new options for pentatomid pest management. This work also developed protocols and procedures useful for researchers interested in caeca-associated symbionts of other insects in the suborder Heteroptera, such as Coreidae and Lygaeidae.

LITERATURE CITED

Abe, Y., Mishiro, K., and M. Takanashi. 1995. Symbiont of brown-winged green bug *Plautia stali* Scott. Jpn. J. Appl. Entomol. Zool. 39: 109-115.

Baumann, P. 2005. Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol 59: 155-189.

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

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers, New York.

Chen, D.Q., Montllor, C.B., and A.H. Purcell. 2000. Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfafa aphid, *A. kondoi*. Entomol. Experim. Appl. 95: 315-323.

Daane, K.M., Yokota, G.Y., Krugner, R., Steffan, S.A., Silva, P.G., Beede, R.H., Bentley, W.J. and G.B. Weinberger. 2005. Large bugs damage pistachio nuts most severely during midseason. Calif. Agricult. 59: 95-102.

Dale, C., and I. Maudlin. 1999. *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. Int. J. S. Bacteriol. 49: 267-275.

Dale, C., Young, S.A., Haydon, D.T., and S.C. Welburn. 2001. The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. Proc. Natl. Acad. Sci. USA. 98: 1883-1888.

Dale, C., Plague, G.R. Wang, B., Ochman, H. and N.A. Moran. 2002. Type III secretion systems and the evolution of mutualistic endosymbiosis. Proc. Natl. Acad. Sci. USA. 99:12397-12402.

Davis, C.J. 1964. The introduction, propagation, liberation and establishment of parasites to control *Nezara viridula* variety *smaragdula* (Fabricius) in Hawaii (Heteroptera: Pentatomidae). Proc. Hawaii Entomol. Soc. 18: 369-375.

Douglas, A.E. 1996. Reproductive failure and the free amino acid pools in pea aphids (*Acyrthosiphon pisum*) lacking symbiotic bacteria. J. Ins. Physiol. 42: 247-255.

Fortes, P., Magro, S.R., Panizzi, A.R., and J.R.P. Parra. 2006. Development of a dry artificial diet for *Nezara viridula* (L.) and *Euschistus heros* (Fabricius) (Heteroptera: Pentatomidae). Neotrop. Entomol. 35: 567-572.

Fukatsu, T., and T. Hosokawa. 2002. Capsule-transmitted gut symbiotic bacterium of the japanese common plataspid stinkbug, *Megacopta puctatissima*. Appl. Environ. Microbiol. 68: 389-396.

Hosokawa, T. Kikuchi, Y. Meng, X.Y. and T. Fukatsu. 2005. The making of symbiont capsule in the plataspid stinkbug *Megacopta punctatissima*. Fems Microbiol. Ecol. 54: 471-477.

Jones, V.P., and L.C. Caprio. 1990. Biology and control of insect pests attacking macadamia nuts in Hawaii. Proc. Hawaii Macadamia Nut Assoc. 30: 24-36.

Kikuchi, Y., Hosokawa, T., and T. Fukatsu. 2007. Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. Appl. Environ. Microbiol. 73: 4308-4316.

Kikuchi, Y. Meng, X. Y. and T. Fukatsu. 2005. Gut symbiotic bacteria of the genus Burkholderia in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Heteroptera: Alydidae). Appl. Environ. Microbiol. 71: 4035-4043.

Koga, R., Tsuchida, T., and T. Fukatsu. 2003. Changing partners in an obligate symbiosis: a facultative endosymbiont *Buchnera* in an aphid. Proc. R. Soc. Lond. B. 270: 2543-2550.

Legaspi, J.C. 2004. Life history of *Podisus maculiventris* (Heteroptera: Pentatomidae) adult females under different constant temperatures. Environ. Entomol. 33: 1200-1206.

Matthew, C.Z., Darby, A.C., Young, S.A., Hume, L.H., and S.C. Welburn. 2005. The rapid isolation and growth dynamics of the tsetse symbiont *Sodalis glossinidius.* FEMS Microbiol. Lett. 248: 69-74.

Montllor, C.B., Maxmen, A., and A.H. Purcell. 2002. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecol. Entomol. 27: 189-195.

Ohtaka, C., and H. Ishikawa. 1991. Effects of heat treatment on the symbiotic system of an aphid mycetocyte. Symbiosis 11: 19-30.

Oliver, K.M., Moran, N.A., and M.S. Hunter. 2005. Variation in resistance to parasitism in aphids is due to symbionts and not to host genotype. Proc. Nat. Acad. Sci. Unit. Sta. Amer. 102: 12795-12800.

Panizzi, A.R., McPherson, J.E., James, D.G., Javahery, M., and R.M. McPherson. 2000. Economic importance of stink bug (Pentatomidae). In Schaefer C.W. and A.R. Panizzi [eds.], Heteroptera of economic importance, CRC, Boca Raton, Florida.

Russell J.A., and N.A. Moran. 2006. Cost and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proc. R. Soc. Lond. B. 273: 603-610.

Scarbourough, C.L., Ferrari, J., and H.C. Godfray. 2005. Aphid protected from pathogen by endosymbiont. Science. 310: 1781.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. and H. Ishikawa. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. Nature 407: 81-86.

Thao, M. L., P. J. Gullan, and P. Baumann. 2002. Secondary (gamma-*Proteobacteria*) endosymbionts infect the primary (beta-*Proteobacteria*) endosymbionts of mealybugs multiple times and coevolve with their hosts. App. Environ. Microbiol. 68: 3190-3197.

Vivan L.M., and A.R. Panizzi. 2005. Nymphal and adult performance of genetically determined types of *Nezara viridula* (L.) (Heteroptera: Pentatomidae), under different temperature and photoperiodic conditions. Neotrop. Entomol. 34: 911-915.

Zanuncio, T.V., Zanuncio, J.C., Serrao, J.E., Medeiros, R.S., Pinon, T.B.M., and C.A.Z. Sediyama. 2005. Fertility and life expectancy of the predator *Supputius cincticeps* (Heteroptera: Pentatomidae) exposed to sublethal doses of permethrin. Biol. Res. 38: 31-39.

CHAPTER 2

VERTICAL TRANSMISSION OF A PENTATOMID CAECA-ASSOCIATED SYMBIONT

Simone de Souza Prado

ABSTRACT: We present molecular data for an endosymbiont of the insect family Pentatomidae, located in the gastric caeca of *Nezara viridula* (L.) stink bugs. Restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) analysis suggest that this bacterium is consistently present in caeca of *N. viridula* from a variety of geographic locations. The bacterium is present in different midgut sections in nymphs versus adults. The bacterium was also detected on eggshells after nymphs had hatched but not in ovarioles, suggesting oral rather than transovarial transmission. Surface sterilization of egg masses generated aposymbiotic insects. Aposymbiotic individuals reached the adult stage, laid viable eggs, and their offspring remained aposymbiotic in the following generation. No clear fitness decrease was observed in aposymbiotic individuals over two generations. Phylogenetic analysis of a partial 16S rRNA dataset with 21 Gammaproteobacteria suggested the inadequacy of neighbor-joining and maximum parsimony models to account for homoplasy apparent in a molecular dataset including a range of insect endosymbionts. Maximum likelihood based analysis suggests that the *N. viridula* endosymbiont is closely related to a caeca-associated symbiont found in another stinkbug family (Plataspidae). The high AT content of the symbiont's 16S rRNA in relation to other insect endosymbionts, its location in the midgut of the host insect, oral transmission and survival of aposymbiotic individuals, suggest this symbiosis may be recently established.

2.1 INTRODUCTION

The association between insects in the order Hemiptera and their bacterial symbionts has long been of interest to entomologists and microbiologists. Much work has focused on the suborders Sternorrhyncha and Auchenorrhyncha, most of which feed on nutrient-poor diets, such as phloem or xylem sap. For these insect hosts bacterial symbionts provide nutrients lacking in their diet (Ishikawa 2003).

Among the various insect-microbe symbiotic interactions studied with hemipteran hosts, the symbiosis between aphids and bacteria of the genus *Buchnera* (Munson) has attracted extensive attention (Koga et al. 2003, Moran 2003, Tamas et al. 2002). Transovarial transmission is a common characteristic of the biology of symbionts in both auchenorrhynchans (e.g. leafhoppers, planthoppers) and sternorrhynchans (aphids, whiteflies, psyllids and mealybugs) (Buchner 1965). This vertical mode of transmission generates a high level of dependency between host and symbiont (Douglas 1996, Wernegreen 2002).

In contrast, oral transmission is the strategy used by many bacterial symbionts of the suborder Heteroptera (true bugs) Buchner (1965). Mechanisms for oral transmission of microbial symbionts include coprophagy (Beard et al. 2002), ingestion of bacteria-filled capsules associated with egg masses (Fukatsu and Hosokawa 2002), and probing on the surface of eggs for symbionts smeared by females (Abe et al. 1995, Buchner 1965). This transmission strategy adds more challenges to the symbiotic association than the transovarial method because the bacterium must survive in an alternate environment outside the host (e.g., excrement or egg surface), and colonize an insect that might already have acquired other microbes from the environment. In some cases, orally transmitted microbes can be cultured *in vitro* (Durvasula et al. 1997), suggesting the presence of more complete metabolic pathways than *Buchnera* or other endosymbionts which are vertically transmitted and to date cannot be cultured (Moran 2003).

While advances have been made in recent years in our understanding of the biology of symbionts associated with various sternorrhynchans and blood-feeding heteropterans of medical importance, little is known about the caeca-associated symbionts of their plant-feeding heteropteran counterparts. Fukatsu and Hosokawa (2002) studied the microbes associated with the gastric caeca of *Megacopta punctatissima* Montandon, a stink bug in the family Plataspidae. *Megacopta punctatissima* females lay eggs and symbiont-filled capsules, and hatching nymphs probe the capsules to acquire the symbiont. Physical removal and heat treatment of capsules impaired nymphal development (Fukatsu and Hosokawa 2002). The proposed transmission mode of symbionts for pentatomids (egg smearing) is different from plataspid bugs, but the general organization of the alimentary tract and caeca in which they are present is similar (Buchner 1965, Goodchild 1963). As with plataspids, elimination of caeca-associated symbionts (by surface sterilization of egg masses) was shown to negatively affect pentatomid fitness (Abe et al. 1995, Buchner 1965).

Although bacteria have been known to be associated with the gastric caeca of Pentatomidae since the late 1800s, these microbes have not been identified. The first comprehensive work on this topic was done by Glasgow (1914). He observed

that although bacteria from caeca of different hosts were morphologically different, bacterial morphology was constant within each host species, and they were always present in a monoculture. Those results led Glasgow to suggest that these bacteria were adventitious, and that their major function was to exclude/inhibit other microbes from multiplying in the caeca. Kuskop (1924) and Rosenkranz (1939), cited by Buchner (1965), suggested that these bacteria had symbiotic roles related to insect nutrition. Rosenkranz (1939) introduced the hypothesis that transfer of symbionts occurred orally, suggesting that females coated their eggs with bacteria, which were acquired by nymphs after hatching.

Despite the large number of studies on the identification and phylogenetic placement of hemipteran endosymbionts among other bacteria, the position of many taxa is inconclusive (Aksoy 2003, Fukatsu and Nikoh 1998, Thao et al. 2002). Variability in phylogenetic associations may be due to the taxa used in the analysis or to methods of tree construction which can lead to inconsistent trees (Brocchieri 2001). Both distance-based and maximum parsimony (MP) methods have been shown to be vulnerable to homoplasy when the data sets cover relatively distantly related groups of organisms and deeper time divergences (Herbeck et al. 2005), probably due to substitution saturation of base pairs and possible nucleotide heterogeneity. Longer phylogenetic branches 'attract' each other leading to unification or proximity of taxa that are actually not closely related (Felsenstein 1978, Graybeal 1998, Hillis 1998). This homoplasy is exacerbated by nucleotide heterogeneity between taxa in analyses (Steel et al. 1995, Galtier and Gouy 1998, but see Conant and Lewis 2001, Rosenberg and Kumar 2003). Endosymbiont systematic studies frequently use distance and parsimony-based models, which are vulnerable to this phenomenon (Herbeck et al. 2005). Maximum likelihood (ML) is able to more effectively analyze datasets with different evolutionary rates, as is the case for datasets in the Gammaproteobacteria that include insect endosymbionts (Moran 1996). An additional confounding factor in sequence analysis may be that the alignments of 16S rRNA sequence differ in length, and most studies eliminate any sequence data that is not represented in all of the taxa in an analysis. Such a 'no-gaps' data matrix makes all taxa of equal sequence length, but eliminates large portions of data.

We identified a bacterium molecularly that is consistently associated with the caeca of *Nezara viridula* (L.) (Hemiptera, Pentatomidae), an economically important and cosmopolitan pest of various crops. To phylogenetically place this bacterium and to assess the potential impact of homoplasy on endosymbiont phylogenetic reconstruction, we compared the results from distance and parsimony models with an optimized ML model designed to better account for homoplasy (Huelsenbeck 1997, Posada and Crandall 1998). We also showed that aposymbiotic insects can be generated after surface sterilization of egg masses.

2.2 MATERIALS AND METHODS

2.2.1 Insects

We established a *N. viridula* laboratory colony in the summer of 2003 with adults collected from legumes growing adjacent to a macadamia farm in Hilo, Hawaii, USA. We occasionally added other individuals, collected at the same area, to the colony. Insects were reared in plastic containers in an insectary room with controlled temperature (25 ± 2°C). Green beans and peanuts were used as diet (Todd 1989). *Nezara viridula* adult females from California, South Carolina and Florida were used to determine if the bacterium was present in geographically distinct insect populations.

2.2.2 Transmission electron microscopy

The last section of the midgut (V4; Figure 2.6.1) of second instars was dissected for transmission electron microscopy. We fixed the tissue in 4% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.45). Samples were post fixed in 1% OsO4 in 0.1M cacodylate buffer, dehydrated through a graded series of alcohol, substituted with propylene oxide, then infiltrated and embedded in LX-112 epoxy resin (Ladd). Ultrathin sections were taken on a Reichert Ultracut E ultramicrotome and photographed on a LEO 912 EFTEM at 80kV with a Proscan frame-transfer CCD.

2.2.3 PCR, RFLP, and sequencing

We used standard procedures for all experiments unless noted. We dissected ten adult females under a microscope, extracted the gut, and transferred the last portion of the midgut with 4 rows of gastric caeca (segment V4; see below for description) to a tube with sterile phosphate buffered saline (PBS). Samples were washed multiple times with PBS to eliminate surface contaminants and ground with a plastic pestle. DNA was extracted with a commercial DNA extraction kit (Promega Corp, Madison, WI #A1120). Primers 16SA1 and 16SB1 were used for amplification of a fragment of the 16S rRNA gene from bacteria present in samples as previously described (Fukatsu and Nikoh 1998). PCR products were ligated into vector pGEM-T Easy (Promega Corp, Madison, WI #A1360). *Escherichia coli* DH5α was transformed with plasmids and mutants selected on solid media with ampicilin (70 µg/mL). Clones were grown overnight at 37°C on LB liquid medium with ampicilin, and plasmids extracted (Qiagen, Valencia, CA #27104). We amplified the inserts using flanking sequences of the vector as primers (T7 and Sp6 promoters) and determined fragment size. Restriction enzymes *Rsa*I, *Dde*I, *Sau*3AI, and *Taq*I were used for RFLP analysis of the insert. Sequencing was done for 8 clones (clones obtained from different

individuals) with plasmids as template, using 16SA1 and 16SB1 primers initially, then with internal primers designed based on the preliminary reads obtained. Sequencing was done at the Greenwood Molecular Biology Facility (Pacific Biomedical Research Center, University of Hawaii at Manoa, Honolulu HI).

2.2.4 PCR detection of symbiont

The forward primer 16S-PNv (5'-GCCTAATATGCATGATC-3') was designed for *N. viridula*'s caeca symbiont based on sequences obtained and sequence comparisons with other bacteria, and used with the reverse primer 16SB1 (Fukatsu and Nikoh 1998) for PCR amplification of a ~1Kb fragment of the 16S rRNA gene of the symbiont. The temperature profile was: 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 53 °C for 1 min, 71 °C 2 min and one cycle at 71 °C for 5 min. We compared detection of bacteria in caeca and *N. viridula*'s thorax muscles, leafhoppers (*Macrosteles* sp. (Hamilton)) and fruit flies (*Bactrocera* sp). To determine the location of the bacterium in the midgut of individual insects, we used ten other adult females from our colony and dissected them in sterile PBS. The midgut and ovarioles were transferred to a clean glass slide and samples washed multiple times during and after dissection to reduce superficial bacterial contamination. Tissue was cut and transferred to 100µL of PBS; dissected samples included ovarioles and midgut sections V1, V2, V3 and V4, all individually tested (Figure 2.6.1; Silva and Terra 1994). We also dissected ten 4th instars of *N. viridula*, but midgut sections V1, V2 and V3 were combined into one sample, and V4 was tested separately. In this case V1+V2+V3 were separated from V4 by cutting the constriction tissue separating these sections of the midgut. DNA from samples was extracted as previously described.

2.2.5 Phylogenetic analysis

For the phylogeny we selected bacteria that included primary and secondary endosymbionts from a range of families of sap-sucking Hemiptera and other insect orders, as well as free-living bacteria within the Enterobacteriaceae (Table 2.5.1). One clone of *N. viridula* was used for our phylogenetic analysis. All other bacterial nucleotide data were downloaded from Genbank (Table 2.5.1). Sequences were aligned with ClustalW v.1.83 (Chenna et al. 2003), and manually checked with MacClade v.4.05 (Maddison and Maddison 2002). Bacterial sequences used for analysis ranged from 1377 to 1450 bases (Table 2.5.1). After alignment, all sequences were cut at the first and last bases of *N. viridula*. All phylogenetic analyses were conducted using PAUP* 4.0b10 (Swofford 2002). Distance (Neighbor-Joining- NJ) and MP searches were performed using all defaults. ModelTest v.3.5 (Posada and Crandall 1998) was used to optimize a ML model for the data set with the Akaike Information Criterion for both the 'gaps' and 'no gaps' data sets (see below). PAUP

was used to implement the models recommended by ModelTest under a ML analyses. Bootstrap values for NJ, MP and ML analyses (1000 replicates) were calculated in PAUP*, decay index values were performed using TreeRot (Sorenson 1999).

The effects of retaining or eliminating gaps from the molecular dataset were also investigated using identical search methods for a 'gaps' and 'no gaps' data set. Gaps, genomic regions in which insertions/deletions have occurred for some of the taxa in an analysis, are often removed during sequence alignment of bacterial 16S rRNA. To test the importance of gaps in our dataset, we conducted identical phylogenetic analyses on datasets for which we removed nucleotides for all taxa in which one or more taxa had insertion/deletions ('no gaps') and compared the results to datasets in which we left gaps as they occurred in the sequence ('gaps'). For the 'gaps' dataset, gaps were coded as missing data. All trees were rooted with *Pantoea agglomerans* (Ewing and Fife) and *Erwinia herbicola* (Lohnis), because they were hypothesized to represent likely outgroups for the other bacteria chosen for the analyses.

2.2.6 Surface sterilization of egg masses

We surface sterilized eggs by dipping them for 5 minutes into 95% ethanol followed by 5 minutes in a 10% bleach solution. Eggs were allowed to air dry and nymphs hatched normally. We sterilized eggs approximately 4-5 days after oviposition; nymphs usually hatched within 6 days. Twenty egg masses were used for this experiment; each one was divided into two halves, one non-treated (control group) and the other surface sterilized (treated group). All hatching nymphs were allowed to develop to adulthood, and maintained in individual containers. For each of the cohorts we randomly selected insects and sampled the following (by PCR) for presence of the symbiont: a) egg mass after nymphs hatched, b) two 5th instars (V4), c) one adult male (V4) and d) one adult female (V4). In a few cohorts we had high mortality or smaller number of insects available, thus the total number of samples tested was smaller than the initial numbers might suggest. Adults from this first generation were combined into a cage (treatments separated) and allowed to mate and lay eggs. If any individual of a cohort in the first generation tested positive for the symbiont, the entire cohort was excluded from the mating cage. Offspring (second generation, from12 symbiotic and 9 aposymbiotic egg masses) were reared to adulthood and sampled for presence of the symbiont as described for the first generation. We also counted, for both generations, the day the first nymph of each cohort molted into the next instar. We did not identify these individuals, thus our observations may have been made on different individuals from the same cohort.

2.3 RESULTS

2.3.1 Midgut organization

The midgut of a female adult *N. viridula* can be divided into 4 sections (ventricula): V1, V2, V3 and V4; gastric caeca are associated with V4 (Figure 2.6.1). These four regions are similar to those observed in other Pentatomidae (Goodchild 1963, Silva and Terra 1994). However, when 2nd, 3rd, 4th and 5th instars were dissected, all had a sac-like section constricting the flow of ingested food from V3 to V4 (data not shown). This constriction was not observed in adults. Transmission electron microscopy sections of V4 showed the presence of bacterial rods in the V4 section of the midgut of nymphs (Figure 2.6.2).

2.3.2 RFLP and sequencing

Forty-two of 45 clones had the expected ~1.5 Kb insert (checked by PCR); all 10 insects were represented by at least 1 clone but no more than 5 clones. All 42 inserts had the same RFLP profile, with only one exception (an insert with sequence similarity to the genus *Brevibacterium*, which was eliminated from further analysis. Clones from different individuals were sequenced on both strands, and the partial 16S rRNA sequence (1416 bp) was identical for all clones (with the exception of four nucleotides, assumed to be amplification errors). One representative sequence was selected for phylogenetic analysis (AY679762).

2.3.3 PCR detection of symbiont

All DNA extracts from V4 yielded a single band of about 1Kb after PCR, as expected. DNA extracted from *N. viridula* thoracic muscle did not generate a PCR amplicon using the same primers. The primer set did not generate a product when template DNA from leafhoppers or fruit flies was used. We also digested amplicons with restriction enzymes as described above. Digests matched the results expected when NEBcutter 2.0 (http://tools.neb.com/NEBcutter2/index.php) was used with the sequence from the 16S rRNA fragment expected to be amplified by the primers. Insects from California were tested for the presence of the symbiont using these primers (n = 20 individuals); all those insects were positive. Some of the amplicons were subjected to RFLP and these samples had the expected profiles. We also sequenced the amplicon of individuals (V4 gut section) collected in California, South Carolina, Florida and a macadamia farm in Hilo, HI. Amplicon sequences (~1KB) were identical to our initially obtained sequence. We also retrieved sequence AY830409 (16S rRNA) from GenBank, obtained from the midgut of *N. viridula* (location of insect collection not identified), which was also identical to that of our bacterium. These results suggest that this bacterium is consistently associated with the V4 section of *N. viridula*'s midgut regardless of geographic location.

We detected the symbiont in different sections of the midgut of *N. viridula* adults as compared to nymphs (Table 2.5.2). Symbionts occurred in both V3 and V4 sections of the adults, and in one case we observed faint bands for V1 and V2 as well; the latter may have been surface contaminants or low titers of bacteria in these anterior sections of the midgut. Symbionts were only detected in V4 of nymphs, however, and none of the tested individuals was positive for any of the three other anterior sections of the midgut (Figure 2.6.3).

2.3.4 Phylogenetic analyses

No gaps dataset. The NJ model under distance parameters gave a tree that was different from the parsimony consensus tree only in its level of resolution, but not in any relationships between taxa (Figure 2.6.4). Most interesting perhaps, is the placement of the *N. viridula* symbionts branching basally or unresolved in relation to a clade containing other endosymbionts. Under the MP model, the data set for which gaps were eliminated yielded three most parsimonious trees of 1106 steps, with 265 characters that were parsimony informative. The strict consensus of the three trees suggests a single, poorly resolved, origin for hemipteran endosymbionts (Figure 2.6.4). ModelTest chose a general time reversible model with a gamma distribution and a portion of invariable sites estimated from the data (best-fit model =GTR+I+G). The ML tree (Figure 2.6.4), likelihood score –ln(L)=7082.07704, differed from the MP and NJ trees and suggests two independent origins for hemipteran primary endosymbionts, with the *N. viridula* endosymbiont in a clade containing *M. punctatissima* and *Buchnera*.

Gaps dataset. For the data set in which all gaps were retained there was a single best MP tree of 1288 steps (Figure 2.6.4), with 300 parsimony informative characters. It differed from the no gaps tree in that the topology was better resolved (one best tree versus three for no gaps) for most terminal taxa, but the two trees did not conflict. The NJ analysis with gaps and no gaps have differences in the placement of the *N. viridula* symbiont, but they were slight and poorly supported by the bootstrap analysis (Figure 2.6.4). ModelTest suggested the same GTR+I+G model as for the no gaps data set. The ML analysis gave a tree that was identical to the ML analysis under the no gaps data set, with a likelihood score of –ln(L)= 8013.34941.

2.3.5 Surface sterilization of egg masses

Nymphs hatching from surface-sterilized eggs were mostly free of the symbiont, as were adults that developed from these nymphs (G1) (Figure 2.6.5). A high percentage of treated egg masses in this first generation, however, were positive for the bacterium. The symbiont was transferred to the second generation (G2) of the *N. viridula* control

cohorts, but not to G2 of the treated cohorts. The absence of the symbiont in a few individuals from control cohorts may be due to incomplete vertical transmission of the bacterium or detection limitations. We found no difference in the number of days required for the first nymph in each cohort to molt to its subsequent instar in the different treatments studied (Figure 2.6.6).

2.4 DISCUSSION

We have molecularly identified a bacterial symbiont associated with the caeca of *N. viridula*. This bacterium is found in Hawaii, California and South Carolina stink bug populations, and may be the major bacterium found in the midgut section with caeca (V4) of this insect. The 16S rRNA partial sequence of the symbiont indicates that it is an Enterobacteriaceae. The sister clade in our ML analysis contains the symbiont of another hemipteran, in the family Plataspidae (Fukatsu and Hosokawa 2002). Our experiments strongly suggest that transmission occurs orally rather than transovarially. Surface sterilization of eggs generated aposymbiotic insects and aposymbiotic individuals survived well in our laboratory conditions.

The presence of the bacterium in the V3 and V4 sections of the midgut of adult females but in only V4 in 4th instars, suggests a difference in gut morphology between adults and nymphs, as previously reported (Goodchild 1966). These results support previous reports that there is a blockage in the alimentary canal of pentatomids between V3 and V4 midgut sections in nymphs, but not in adults (i.e. bacteria also present in V3 of adults). In this study, dissection of 2nd to 5th instars of *N. viridula* showed the presence of a blister between these sections in all insects analyzed (data not shown). Detection of the bacterium in V3 in adults may have occurred after movement of cells during dissection rather than natural placement. It is unclear whether 1st instars have the restriction between V3 and V4, but our results raise the question of how bacterial cells reach the V4 region during initial stages of colonization.

Glasgow (1914) demonstrated that the alimentary tracts of pentatomid embryos contained bacteria before hatching, suggesting transovarial transmission of the symbiont. However, Rosenkranz (1939) suggested that transmission does not occur transovarially, but rather when nymphs probe on the surface of eggs after hatching. Lockwood and Story (1986) tested this hypothesis by surface sterilizing egg masses of *N. viridula* with 15% formalin for 20 minutes, and determined that sterilization had no effect on nymphal mortality. Abe et al. (1995) showed that different surface-sterilization treatments resulted in variable development rates for another pentatomid, *Plautia stali* (Scott). Thus, authors studying the effect of surface sterilization of Pentatomidae eggs on insect fitness have conflicting results. We were able to eliminate a caeca-associated symbiont in the majority of *N. viridula*

given access only to surface-sterilized egg masses after hatching. We observed no obvious fitness decrease in those insects over two generations, but we did not measure enough parameters for a conclusive answer regarding the importance of this association. Furthermore, we reared insects under controlled conditions with a constant supply of food. In the future it would be worthwhile to study in detail the development and reproductive rate of aposymbiotic stink bugs under a range of conditions, including limited or unbalanced diets.

Because the partial 16S rRNA dataset used here represents relatively large periods of evolutionary time during which unrelated endosymbionts were exposed to convergent genomic selection, including an AT bias, it is not surprising that there is a strong homoplastic signal pulling all of the endosymbionts into false monophyly. Extreme nucleotide heterogeneity, and the substitution saturation that comes with long divergence times is almost certainly the cause of the 'long-branch' attraction we found with the distance (NJ) and MP models, and the phenomenon has been suggested by other studies (Brocchieri 2001, Huson and Steel 2004). Branch support for the NJ and MP trees is highly variable, and because the bootstrap represents a resampling of a subset of the original data (under the same specified model), it must be interpreted cautiously since it is not a measure of the accuracy of a particular model (Brocchieri 2001). Bootstrapping also assumes independence of nucleotides in the sequence and equal evolutionary rates among taxa, assumptions not valid for 16S rRNA datasets of bacteria that include insect endosymbionts (Moran 1996). Some minor topological differences were observed in analyses with and without gaps. However those occurred primarily as branch swaps in nodes with low statistical support, or as a result of poor resolution within and between basal branching clades, and not taxa. This suggests that the elimination of gaps may not be affecting phylogenetic relationships in our dataset. The placement of the endosymbiont we describe is ambiguous and poorly resolved with NJ and MP analyses. The MP analysis had better resolution than NJ with the dataset that included gaps. This is likely because the no gaps dataset eliminated 35 parsimony informative characters. One of the three most parsimonious trees from the 'no gaps' dataset was congruent with the 'gaps' dataset MP tree. Although the MP and ML trees do not agree, this is probably due to homoplasy in the dataset and is not a function of the elimination of gaps. Resolution and consistency were improved under a ML analysis, likely because the ML is better able to model data with varying evolutionary rates and non-constant levels of AT richness. Concerns about AT-bias and its effect on endosymbiont phylogeny have been raised previously (Charles et al. 2001, Fukatsu and Nikoh 1998). Small AT-rich genomes are probably a response to the similar, simplified, ecological conditions faced by endosymbionts (Herbeck et al. 2003) and therefore represent convergent evolution due to similar evolutionary pressures (Moran 2002).

Few recent studies have addressed the symbiotic flora of plant-feeding heteropterans. Fukatsu and Hosokawa (2002) identified the symbiont of *M. punctatissima* (Plataspidae), which was the bacterium phylogenetically closest to *N. viridula*'s symbiont in our ML analyses. Although these are the only stink bugs for which caeca symbionts have been molecularly identified, differences and similarities between these organisms are worth noting. Both are primarily restricted to the caeca of their host, but one is transferred to the offspring through capsules and the other likely through egg smearing by adult females during oviposition. Although ML phylogenetic analysis suggests that the stink-bug symbionts are closely related, the AT content of the 16S rRNA gene of these bacteria is different (partial sequence analyzed had 45.6 (*N. viridula*) and 50.6% (*M. punctatissima*) AT content). It has been demonstrated that vertically transmitted symbionts have reduced genomes and AT bias (Wernegreen 2002). Similarly, the differences in AT content suggest that an organism transmitted within a capsule, without having to survive and compete on the surface of eggs, may undergo a higher rate of genome degeneration, as found in those bacteria that are transovarially transmitted.

The consistent association with *N. viridula* suggests that this symbiont may provide benefits to its host. However, this system is substantially different from intracellular symbiotic associations of other plant-sucking insects (e.g. aphids and *Buchnera*). The symbiont seems limited to a specific region of the midgut and to be orally transmitted to offspring. Additionally, under laboratory conditions, the host appears to survive and reproduce equally well without the bacterium, suggesting a lower level of dependency between the organisms.

2.5 TABLE

Table 2.5.1. Information on taxa used for phylogenetic analysis.

Bacterium (or insect host[1])	Accession #	Biological information	Host	# sites
Baumannia cicadellinicola (Moran et al.)	AF465793	Primary symbiont	Leafhopper – *Homalodisca coagulata* (Say)	1432
Cand. *Blochmannia rufipes* (Sauer et al.)	X92552	Primary symbiont	Ant – *Camponotus rufipes* (Fabricius)	1450
Buchnera aphidicola– Ap (Munson et al.)	M27039	Primary symbiont	Aphid – *Acyrthosiphon pisum* (Harris)	1421
Buchnera aphidicola– Mr (Munson et al.)	M63255	Primary symbiont	Aphid – *Melaphis rhois* (Fitch)	1436
Wigglesworthia glossinidia (Aksoy et al.)	AB063521	Primary symbiont	Tse-tse fly – *Glossina brevipalpis* (Newstead)	1425
SOPE (Akman et al.)	AF005235	Principal symbiont	Weevil – *Sitophilus oryzae* (L.)	1417
Cand. *Hamiltonella defensa* (Moran et al.)	AF293616	Secondary symbiont	Aphid – *Acyrthosiphon pisum* (Harris)	1422
Sodalis glossinidius (Dale and Maudlin)	AF548136	Secondary symbiont	Tse-tse fly – *Glossina brevipalpis* (Newstead)	1414
[1]*Paratrioza cockerelli* (Spaulding and von Dohlen)	AF286127	Secondary symbiont	Psyllid – *Paratrioza cockerelli* (Sulc)	1417
Cand. *Serratia symbiotica* (Moran et al.)	M27040	Secondary symbiont	Aphid – *Acyrthosiphon pisum* (Harris)	1416
[1]*Megacopta punctatissima*	AB067723	Caeca-associated symbiont	Stink bug – Plataspidae	1420
[1]*Nezara viridula*	AY679762	Caeca-associated symbiont	Stink bug - Pentatomidae	1416
Xenorhabdus poinarii (Akhurst)	X82253	Pathogen/ symbiont	Insect/nematode	1418
Erwinia herbicola (Lohnis)	AF290417	Pathogen	Plant	1407
Pantoea agglomerans (Ewing and Fife)	AJ583011	Pathogen	Plant	1377
Cand. *Phlomobacter betae* (Gatineau et al.)	AY057392	Pathogen	Plant/Insect	1418
Escherichia coli (Migula)	AE000452	Pathogen	Animal	1415
Klebsiella pneumoniae (Schroeter)	AF453251	Pathogen	Animal	1415
Salmonella typhi (Schroeter)	U88545	Pathogen	Animal	1415
Serratia marcescens (Bizio)	AF124042	Pathogen	Animal	1416
Yersinia pestis (Lehmann and Neumann)	AJ232236	Pathogen	Animal	1417

[1] Insect host of bacterial symbiont.

Table 2.5.2. Detection of *Nezara viridula* caeca-associated symbiont in different tissues of adult females and 4th instars.

	Ovarioles	V1[1]	V2	V3	V4
Adult females[2]	0/10	0/10	0/10	9/10	10/10
			V1+V2+V3		V4
4th instars[2]			0/10		10/10

[1] Individual midgut sections sampled

[2] Number of PCR positive samples / Number of individuals tested

2.6 FIGURES

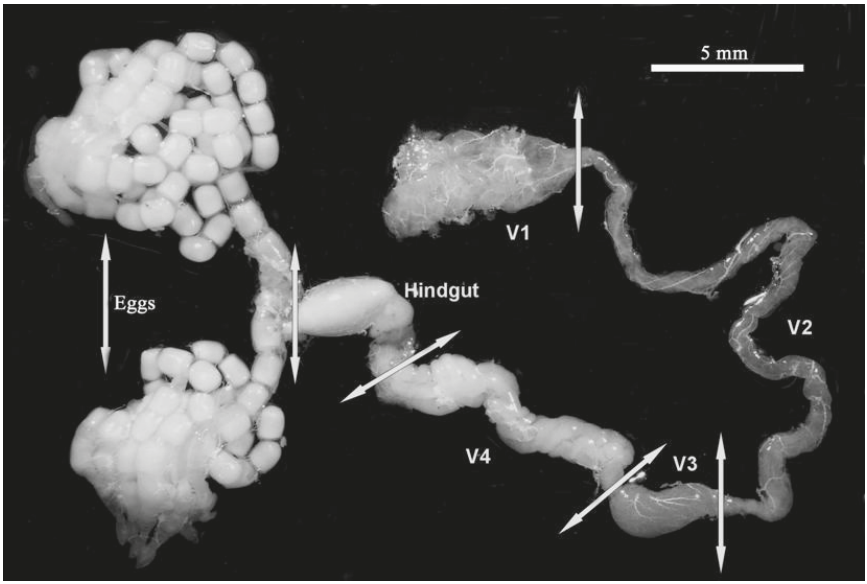


Figure 2.6.1. *Nezara viridula* adult female gut sections relevant to this study. Picture shows different midgut sections (V1-V4), hindgut and eggs/ovarioles. Lines illustrate regions of the midgut that were cut for PCR detection of symbiont.

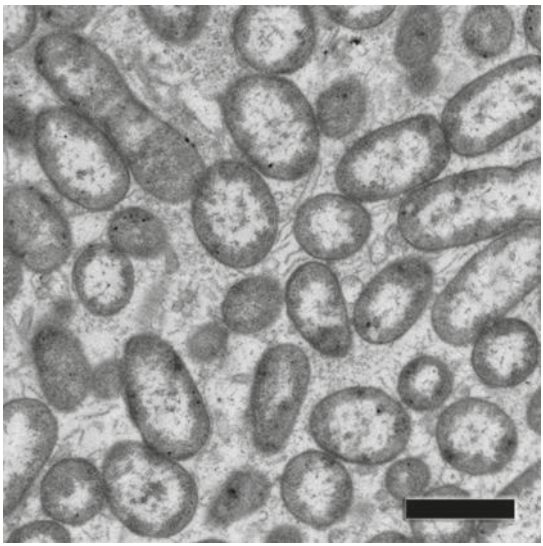


Figure 2.6.2. Transmission electron microscopy cross section of the lumen of a crypt in the V4 section of a second instar *Nezara viridula* shows presence of bacteria. Bar = 1 µm.

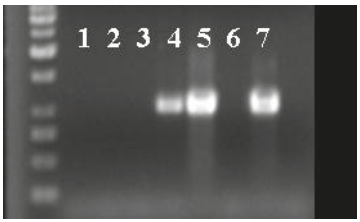


Figure 2.6.3. PCR amplification of partial 16S rRNA gene of caeca-associated symbiont of a dissected adult female (lanes 1-5) and a 4th instar (lanes 6-7) of *Nezara viridula* using caeca-symbiont specific primers. Adult female: lanes 1) ovariole, 2) V1, 3) V2, 4) V3, 5) V4; 4th instar: lanes 6) V1+V2+V3 and 7) V4. Fragments of ~1Kbp, expected with the primer pair used, indicate positive samples.

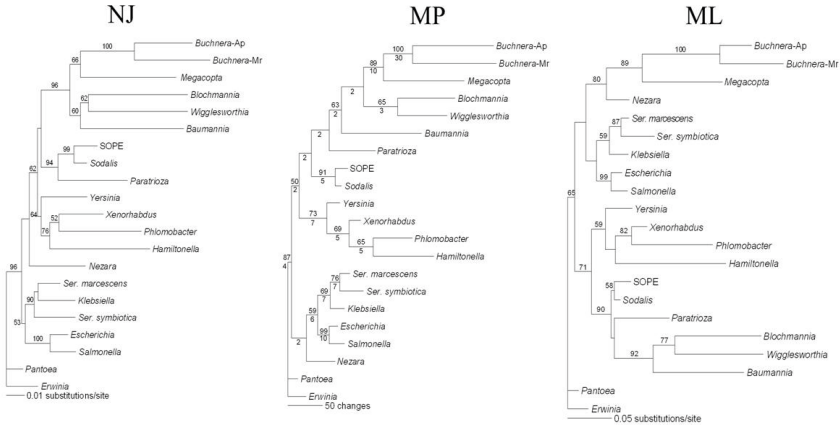


Figure 2.6.4. For all three models, data set used is 'with gaps', clades retained by heuristic searches, but not represented in the 1000 replicate bootstrap consensus, automatically have bootstrap values of <50%. Bootstrap values above nodes, and for MP tree, decay index values below nodes. See Table 2.5.1 for details about sequences used. Note the support for multiple origins for endosymbionts and placement of the *Nezara* symbiont. NJ 'No gaps' data set phylogram was similar except that P-*Nezara* was placed as sister to all other endosymbionts, though this had poor branch support. MP 'No gaps' phylogeny did not conflict but consensus tree was poorly resolved. ML 'No gaps' topology was identical.

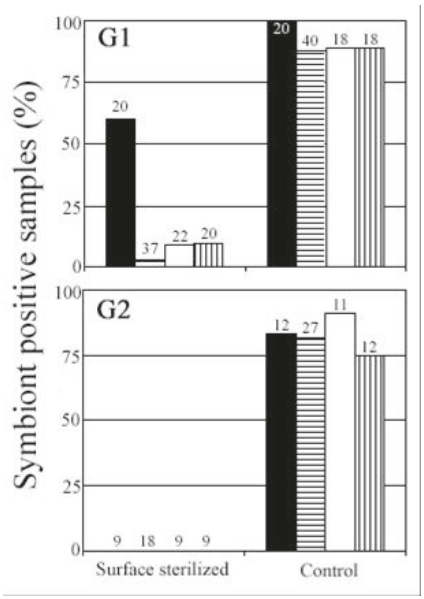


Figure 2.6.5. Effect of egg surface sterilization on the presence of caeca-associated symbiont of *Nezara viridula* over two generations. Eggs were treated only in the first generation (G1). Adults from symbiont-free cohorts of G1 were combined and started a second generation (G2). Numbers on top of bars indicate total number of individuals tested by PCR for symbiont's presence. Black bar – egg mass; bar with horizontal lines – 5th instar V4; white bar – adult male V4; bar with vertical lines – adult female V4.

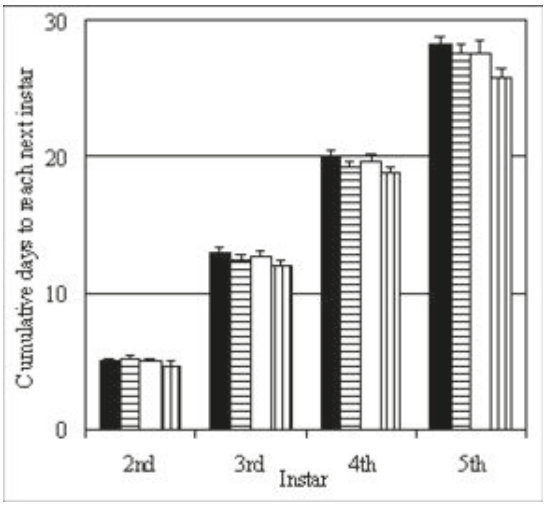


Figure 2.6.6. Mean length of time (cumulative number of days) taken for the first nymph to molt from one stage to the next. Black bar – first generation of surface sterilized eggs; horizontal lines – first generation control; white bar – second generation of surface sterilized eggs; vertical lines – second generation control. Standard error for each treatment shown on top of bars.

LITERATURE CITED

Abe, Y., K. Mishiro, and M. Takanashi. 1995. Symbiont of brown-winged green bug *Plautia stali* Scott. Jpn. J. Appl. Entomol. Zool. 39: 109-115.

Aksoy, S. 2003. Symbiosis in tsetse, pp. 53-65. *In* K. Bourtzis and T. A. Miller [eds.], Insect symbiosis. CRC Press.

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

Brocchieri, L. 2001. Phylogenetic inferences from molecular sequences: review and critique. Theoret. Pop. Biol. 59: 27-40.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers, New York.

Charles, H., A. Heddi, and Y. Rahbe. 2001. A putative insect intracellular endosymbiont stem clade, within the Enterobacteriaceae, inferred from phylogenetic analysis based on a heterogeneous model of DNA evolution. Compt. Rend. l'Acad. Sci. Serie III Sci Vie 324: 489-494.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. Gibson, D. Higgins, and J. Thompson. 2003. Multiple sequence alignment with the Clustal series of programs. Nuc. Acids Res. 31: 3497-3500.

Conant, G. C., and P. O. Lewis. 2001. Effects of nucleotide composition bias on the success of the parsimony criterion in phylogenetic inference. Mol. Biol. Evol. 18: 1024-1033.

Douglas, A. E. 1996. Reproductive failure and the free amino acid pools in pea aphids (Acyrthosiphon pisum) lacking symbiotic bacteria. J. Insect Physiol. 42: 247-255.

Durvasula, R. V., A. Gumbs, A. Panackal, O. Kruglov, S. Aksoy, R. B. Merrifield, F. F. Richards, and C. B. Beard. 1997. Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc. Nat. Acad. Sci.(USA). 94: 3274-3278.

Felsenstein, J. 1978. Cases in which parsimony and compatibility methods will be positively misleading. Syst. Zool. 27: 401-410.

Fukatsu, T., and N. Nikoh. 1998. Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). Appl. Environ. Microbiol. 64: 3599-3606.

Fukatsu, T., and T. Hosokawa. 2002. Capsule-transmitted gut symbiotic bacterium of the japanese common plataspid stinkbug, *Megacopta puctatissima*. Appl. Environ. Microbiol. 68: 389-396.

Galtier, N., and M. Gouy. 1998. Inferring pattern and process: maximum-likelihood implementation of a nonhomogeneous model of DNA sequence evolution for phylogenetic analysis. Mol. Biol. Evol. 15: 871-879.

Glasgow, H. 1914. The gastric caeca and the caecal bacteria of the Heteroptera. Biol. Bull. Woods Hole 26: 101-170.

Goodchild, A. J. P. 1963. Studies on the functional anatomy of the intestines of Heteroptera. Proc. Zool. Soc. London 141: 851-910.

Goodchild, A. J. P. 1966. Evolution of the alimentary canal in the Hemiptera. Biol. Rev. 41: 97-140.

Graybeal, A. 1998. Is it better to add taxa or characters to a difficult phylogenetic problem? Syst. Biol. 47: 9-17.

Herbeck, J. T., P. H. Degnan, and J. J. Wernegreen. 2005. Nonhomogeneous model of sequence evolution indicates independent origins of primary endosymbionts within the enterobacteriales (alpha-proteobacteria). Mol. Biol. Evol. 22: 520-532.

Herbeck, J. T., D. J. Funk, P. H. Degnan, and J. J. Wernegreen. 2003. A conservative test of genetic drift in the endosymbiotic bacterium Buchnera: Slightly deleterious mutations in the chaperonin groEL. Genetics 165: 1651-1660.

Hillis, D. M. 1998. Taxonomic sampling, phylogenetic accuracy, and investigator bias. Syst. Biol. 47: 3-8.

Huelsenbeck, J. P. 1997. Is the Felsenstein zone a fly trap? Syst. Biol. 46: 69-74.

Huson, D. H., and M. A. Steel. 2004. Distances that perfectly mislead. Syst. Biol. 53: 327-332.

Ishikawa, H. 2003. Insect symbiosis: an introduction, pp. 1-21. *In* K. Bourtzis and T. A. Miller [eds.], Insect Symbiosis. CRC Press.

Koga, R., T. Tsuchida, and T. Fukatsu. 2003. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont Buchnera in an aphid. Proc. Roy. Soc. London Series B-Biol. Sci. 270: 2543-2550.

Kuskop, M. 1924. Bakteriensymbiosen bei wanzen. Arch. Protistenk. 47.

Lockwood, J. A., and R. N. Story. 1986. Adaptive functions of nymphal aggregation in the southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae). Environ. Entomol. 15: 739-749.

Maddison, D., and W. Maddison. 2002. MacClade: Analysis of Phylogeny and Character Evolution, Sunderland, Massachusetts: Sinauer Associates.

Moran, N. A. 1996. Accelerated evolution and Muller's rachet in endosymbiotic bacteria. Proc. Nat. Acad. Sci. (USA). 93: 2873-2878.

Moran, N. A. 2002. Microbial minimalism: genome reduction in bacterial pathogens. Cell 108: 583-586.

Moran, N. A. 2003. Tracing the evolution of gene loss in obligate bacterial symbionts. Curr. Opin. Microbiol. 6: 512-518.

Posada, D., and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Rosenberg, M. S., and S. Kumar. 2003. Heterogeneity of nucleotide frequencies among evolutionary lineages and phylogenetic inference. Mol. Biol. Evol. 20: 610-621.

Rosenkranz, W. 1939. Die Symbiose der Pentatomiden (Hemiptera, Heteroptera). Z. Morphol. Ökol. Tiere 36:279–309.

Silva, C. P., and W. R. Terra. 1994. Digestive and absorptive sites along the midgut of the cotton seed sucker bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). Insect Biochem. Molec. Biol. 24: 493-505.

Sorenson, M. D. 1999. TreeRot, version 2. Boston University, Boston, MA. http://mightyduck.bu.edu/TreeRot/index.html.

Steel, M. A., P. J. Lockhart, and D. Penny. 1995. A frequency-dependent significance test for parsimony. Mol. Phylogenet. Evol. 4: 64-71.

Swofford, D. L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Mass. computer program, version By Swofford, D. L.

Tamas, I., L. M. Klasson, B. Canback, A. K. Naslund, A.-S. Eriksson, J. J. Wernegreen, J. P. Sandstrom, N. A. Moran, and S. G. E. Andersson. 2002. 50 million years of genomic stasis in endosymbiotic bacteria. Science. 296: 2376-2379.

Thao, M. L., P. J. Gullan, and P. Baumann. 2002. Secondary (gamma-*Proteobacteria*) endosymbionts infect the primary (beta-*Proteobacteria*) endosymbionts of mealybugs multiple times and coevolve with their hosts. Appl. Environ. Microbiol. 68: 3190-3197.

Todd, J. W. 1989. Ecology and behavior of *Nezara viridula*. Annu. Rev. Entomol. 34: 273-292.

Wernegreen, J. J. 2002. Genome evolution in bacterial endosymbionts of insects. Nat. Rev. Genet. 3: 850-861.

CHAPTER 3

PHYLOGENETIC PLACEMENT OF PENTATOMID STINK BUG GUT SYMBIONTS

Simone de Souza Prado

ABSTRACT: Insect bacterial symbionts are ubiquitous, however only a few groups of host families have been well studied in relation to their associations with microbes. The determination of the phylogenetic relationships among bacteria associated with different species within an insect family can provide insights into the biology and evolution of these interactions. We studied the phylogenetic placement of vertically transmitted bacterial symbionts associated with the posterior midgut (crypt-bearing) region of pentatomid stink bugs (Hemiptera, Pentatomidae). Our results demonstrate that different host species carried one major bacterium in their midgut. Phylogenetic analyses of the 16S rRNA gene sequences obtained from the midgut of stink bugs placed all symbionts in a clade with *Erwinia* and *Pantoea* species, both plant-associated bacteria. Results indicate that symbiont monophyly occurs among recently diverged taxa (e.g. within a genus), but does not occur in the Pentatomidae. Results suggest that these vertically transmitted symbionts are occasionally replaced by other taxonomically similar bacteria over evolutionary time. Our findings highlight how the evolutionary history of hemipteran symbionts in unexplored host families may have unpredictable levels of complexity.

3.1 INTRODUCTION

Insects have a diversity of associations with bacterial symbionts (Buchner 1965). Most well characterized vertically transmitted insect symbioses are associations where one bacterial taxon resides within specialized cells of their respective hosts (Baumman 2005). At the opposite extreme are gut-residing bacteria of various insect groups, such as termites, which have extremely complex microbial communities that assist with the digestion of nutrients. Although advances in molecular biology have allowed for in depth studies of some of these systems, the vast majority of insect-microbe symbiotic associations are yet to be characterized.

The biology of heteropteran (Insecta, order Hemiptera, suborder Heteroptera) symbionts has been documented but remains poorly understood (Buchner 1965). Pentatomorphan insects (e.g. stink bugs, shield bugs, etc) have been reported to carry vertically transmitted symbionts in crypts in the midgut lumen. Buchner (1965) summarized what was known about these systems before the advent of molecular tools, and recent research on the biology of pentatomorphan gut symbionts corroborates earlier work. It has been shown that gut symbionts in the family Plataspidae (shield bugs) are vertically transmitted via symbiont-filled capsules, resulting in strict host-symbiont cospeciation (Fukatsu and Hosokawa 2002, Hosokawa et al. 2006). Studies with another family of true bugs, Alydidae (broad-headed bugs), demonstrated that their gut symbionts belong to the genus *Burkholderia*, but those bacteria do not form a monophyletic group and are acquired every new generation from the environment (Kikuchi et al. 2005, 2007). Therefore, despite the fact that the gut symbionts of both plataspids and alydids colonize a similar environment within hosts, the biology of bacterial transmission to new generations and evolutionary history are strikingly different. In the case of pentatomids (stink bugs, Pentatomidae), gut symbionts are smeared on the surface of eggs by ovipositing females and are vertically transmitted from mother to offspring (Buchner 1965, Prado et al. 2006). However, the physiological role of these bacteria has not been determined and their putative mutualistic relationship with host insects remains controversial (Abe et al. 1995, Buchner 1965, Lockwood and Story 1986, Prado et al. 2006). The egg-smearing strategy may represent an intermediate state between the vertical and environmental transmission of gut symbionts by plataspids and alydids, respectively. Thus, although vertical transmission occurs with the egg-smearing strategy, bacteria are acquired from an environment that may be prone to contamination by or competition with other microbes. If that is the case, one would expect that pentatomid gut symbionts have some degree of cospeciation with their host insects, but also find evidence of horizontal transmission. To determine the phylogenetic placement of pentatomid gut symbionts, we characterized the gut bacterial community of nine species of stink bugs.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial community in the gut of stink bugs

Insects were field collected in California and Hawaii, and reared in the laboratory by our group and two research groups in California (K. Daane and J. Millar, University of California, Berkeley and Riverside, respectively). We kept insects at -80ºC prior to dissection. The species *Euschistus heros* was collected in Brazil (Piracicaba, Sao Paulo State) and shipped to Hawaii in 70% alcohol. Taxa were selected to determine

the relationship of gut symbionts present within pentatomid hosts in a genus (*Chlorochroa*), tribe (Pentatomini) and among tribes in the family (see Figure 3.5.1). We dissected two adult females of each of the nine species studied (except *E. heros*, for which only one female was used) under a dissecting microscope, extracting the midgut and transferring it to a clean glass slide (see Table 3.4.1 for list of species). The posterior section of the midgut (V4, crypt- or caeca-bearing region) was then cut, washed and transferred to a lysis buffer for DNA extraction as previously described (Prado et al. 2006). Only this section of the gut was used for the work described here. We sterilized dissecting tools after each insect by flaming them after ethanol rinses. To reduce the chance of surface contaminants, we rinsed the whole midgut at least 3 times with sterile PBS buffer, and subsequently rinsed only the posterior midgut. We extracted total DNA from the posterior midgut of all individuals with a commercial DNA extraction kit (Qiagen DNeasy, Valencia, CA). The bacterial 16S rRNA gene was partially amplified by PCR using 16SA1 and 16SB1 primers (Fukatsu and Nikoh 1998), and the amplicons were cloned into the pGEMT-easy vector following the manufacturer's instructions (Promega, Madison, WI). We determined the size of cloned fragments by amplifying inserts with flanking primers on the vector. Restriction fragment length polymorphism (RFLP) analysis of amplified products with three restriction enzymes (*Dde, Hinf*I, *Rsa*I) was conducted. Purified plasmids were submitted for sequencing of the 16S rRNA gene inserts of the dominant taxa determined by RFLP analysis at the Greenwood Molecular Biology Facility (Pacific Biomedical Research Center, University of Hawaii at Manoa, Honolulu, HI).

3.2.2 Phylogenetic analyses

The same sequence was obtained for both individuals from the same species sampled. The RDP Classifier (Cole et al. 2005) was used to infer the broader phylogenetic placement of these bacteria. After it was determined that the bacterial sequences obtained belonged to the Enterobacteriaceae, a preliminary phylogenetic analysis (maximum parsimony and likelihood searches) with 42 species of enterobacteria was conducted (data not shown). Based on these results, a second dataset was chosen for a more detailed analysis of the phylogenetic placement of pentatomid gut symbionts; *Vibrio harveyi* was used as an outgroup. Initial alignment of 16S rDNA sequences was done using the NAST alignment tool available at Greengenes (http://www.greengenes.lbl.gov) (DeSantis et al. 2006). We used SeqMan II (Lasergene v5, DNASTAR, Madison, WI) to manually check the alignment. We searched all our sequences for the presence of chimeras using Bellerophon (Huber et al. 2004) and the Ribosomal Database II Chimera Check (Cole et al. 2005). No chimeras were detected. Maximum parsimony analysis was conducted with PAUP* 4.0b10 (Swofford 2002) with 1,000 bootstrap replicates. Modeltest 3.7 (Posada and Crandall, 1998)

was used to select a likelihood model (TIM+I+G model using the Akaike Information Criterion framework) for the maximum likelihood analysis, which we ran with PAUP*, with 250 bootstrap replicates for branch support. We also performed a Bayesian inference with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), using MrModeltest 2.2 (Nylander 2004) for model selection. Posterior probability support for nodes were based on 7,500 trees (search parameters – 5,000,000 generations, burnin=2,500, samplefreq=500, 4 chains, GTR+I+G model selected by MrModeltest with Akaike Information Criterion). We used BioEdit 7.0.4 (Hall 1999) to build a 16S rDNA sequence similarity matrix including several *Erwinia* and *Pantoea* taxa, in addition to sequences obtained here, to determine the degree of similarity among sequences, alignment was trimmed and insertions and deletions deleted prior to analysis. The obtained sequences have been deposited in GenBank with accession numbers EU72495-EU72503.

3.3 RESULTS AND DISCUSSION

3.3.1 Bacterial community in the gut of stink bugs

The posterior midgut of each stink bug species studied had one dominant bacterial taxon. The presence of other taxa in lower numbers, as determined by RFLP, may be the result of surface contaminants or the presence of other less abundant bacteria in that gut region (Table 3.4.1). The dominant operational taxonomic unit (OTU) recovered was considered to be the main bacterium associated with its respective host stink bug species, and the only ones used for the phylogenetic analyses. Although all the OTUs from different species were most closely related to plant-dwelling bacteria in the genera *Erwinia* and *Pantoea*, there was moderate 16S rRNA gene sequence similarity with other species in those genera (Table 3.4.2). Following the general guideline that less than 98.7-99% 16S rDNA similarity is indicative of a different bacterial species (Stackebrandt and Ebers 2006), these gut bacteria would likely represent new undescribed species.

Recently, studies on three insect families (Plataspidae, Alydidae, and Pentatomidae) within the hemipteran suborder Heteroptera (true bugs) have shown that the posterior midgut is colonized by one dominant bacterium (Fukatsu and Hosokawa 2002, Kikuchi et al. 2005, Prado et al. 2006). A sac-like structure, or blister, appears to precede this gut region in nymphs of pentatomids, and nymph and adult plataspids (Fukatsu and Hosokawa 2002, Prado et al. 2006). This physical blockage may explain how these orally acquired (after hatching) symbionts occur in what seems to be a monoculture. However, it raises interesting questions about the initial stages of symbiont colonization of the gut; specifically, how do these bacteria reach this region of the gut and how do host insects select for these organisms while eliminating others from this environment?

3.3.2 Phylogenetic analyses

Because our sequences had moderate similarity to published sequences in databases, we first used the RDP Classifier (Cole et al. 2005) to infer the broader phylogenetic placement of these bacteria. All pentatomid gut bacteria were found to be closely related to *Erwinia* and *Pantoea*, both plant-associated genera. We conducted an analysis with pentatomid gut bacteria, various *Erwinia* and *Pantoea* species, and closely related taxa. Tree topology and branch support was similar with maximum parsimony (six most parsimonious trees were obtained), maximum likelihood, and Bayesian analysis (Figure 3.5.1). All gut bacteria were placed in a strongly supported clade with *Erwinia* and *Pantoea* species. However, placement within that clade was variable. Symbionts were placed among *Erwinia* and *Pantoea* species, in three cases with strong branch support (except *E. heros*). The lack of monophyly is evident among taxa as is the support for separate clades with symbionts. We observed monophyly for the genus tested, *Chlorochroa* spp., and for taxa in the tribe Antestiini – *P. stali* and *T. pallidovirens*. However, no monophyly was observed for individuals in the tribe Pentatomini. These results suggest that i) this symbiotic relationship originated multiple times or ii) symbiont replacement occurred in this insect family. Because other heteropteran bugs also have a crypt-bearing posterior midgut and harbor symbionts in a monoculture, we consider the symbiont replacement hypothesis more plausible and parsimonious than the multiple origins one. However, studies with larger number of taxa that include phylogenetic analysis of host insects are necessary to better support this hypothesis. The fact that all symbiont clades identified here are phylogenetically related to *Erwinia* and *Pantoea* suggests that only closely related bacterial taxa are physiologically acceptable to hosts or capable of sustaining infections over generations. The lack of strict monophyly, coupled with host association with bacteria of limited phylogenetic diversity (two genera in this case) resembles the association of alydids with *Burkholderia* (Kikuchi et al. 2007).

The strategy for transmission of gut symbionts in plataspids allows for strict vertical transmission of symbionts, which contrasts with the environmental acquisition of *Burkholderia* in alydids. The vertical transmission of pentatomid symbionts by egg smearing may represent an intermediate state between the transmission strategies of these two other families. Our phylogenetic analysis supports that hypothesis. We found pentatomid symbionts were polyphyletic, but monophyletic in more recently diverged taxa (*Chlorochroa* genus). It may be interesting for future studies to consider the evolutionary forces responsible for the establishment and maintenance of these three distinct bug symbioses based on: i) strict vertical transmission (plataspids), ii) vertical transmission with possible symbiont replacement (pentatomids) and iii) acquisition of symbionts from the environment (alydids).

Our results, combined with those cited above on plataspid and alydid midgut symbionts, show that the posterior region of the midgut of pentatomorphans in different families is colonized by one dominant bacterial taxon, and that insects in these families are dependent on these symbionts for survival (Abe et al. 1995, Buchner 1965, Fukatsu and Hosokawa 2002, Kikuchi et al. 2007, Prado and Almeida, unpublished data). However, a few studies with pentatomids suggest that gut symbionts do not provide clear fitness benefits to host insects (e.g. Lockwood and Story 1986, Prado et al. 2006). This variability in the Pentatomidae-symbiont studies may be hypothesized to occur due to the specific bacterium associated with different host species (potential for variable types of association), or experimental diets used for fitness tests. In addition, the age of the association may be an indication of the degree of host-bacterium mutualism; the longer the association the higher the degree of mutual reliance. Together, these studies suggest heteropteran symbiotic associations are interesting systems not only for the study of mutualistic interactions, but also for comparative studies of gut bacterial infections.

3.4 TABLES

Table 3.4.1. List of pentatomid species studied, including the number of taxa identified by RFLP analysis of cloned 16S rRNA gene, and GC content.

	RFLP of cloned 16S rDNA[1]	
Host species	Dominant OTU[2]	Other OTU
Acrosternum hilare	44-44-44[3]	4-4-4
Chlorochroa ligata	44-44-42	0-0-2
Chlorochroa sayi	44-44-44	1-1-1
Chlorochroa uhleri	43-45-45	2-0-0
Euschistus heros	23-23-23	1-1-1
Murgantia histrionica	34-34-34	1-1-1
Nezara viridula	38-40-38	7-5-7
Plautia stali	43-43-43	2-2-2
Thyanta pallidovirens	49-48-25	0-1-0

[1] RFLP performed with three enzymes (*Dde*-*Hinf*I-*Rsa*I)

[2] Operational taxonomic unit.

Table 3.4.2. Sequence similarity matrix of 16S rRNA gene of pentatomid gut symbionts (GS followed by host species) and several *Erwinia* and *Pantoea* species. Accession number for taxa at phylogenetic tree (Figure 3.5.1).

	GS *A. hilare*	GS *C. ligata*	GS *C. sayi*	GS *C. uhleri*	GS *E. heros*	GS *M. histrionica*	GS *N. viridula*	GS *P. stali*	GS *T. pallidovirens*	E. amylovora	E. mallotivora	E. pyrifoliae	E. rhapontici	E. persicina	E. tracheiphila	E. psidii	E. toletana	P. stewartii	P. agglomerans	P. ananatis
GS *A. hilare*	ID																			
GS *C. ligata*	0.941	ID																		
GS *C. sayi*	0.945	0.985	ID																	
GS *C. uhleri*	0.943	0.992	0.987	ID																
GS *E. heros*	0.957	0.966	0.963	0.966	ID															
GS *M. histrionica*	0.930	0.909	0.912	0.909	0.914	ID														
GS *N. viridula*	0.954	0.942	0.947	0.945	0.945	0.927	ID													
GS *P. stali*	0.956	0.971	0.969	0.971	0.974	0.914	0.950	ID												
GS *T. pallidovirens*	0.956	0.969	0.964	0.969	0.973	0.915	0.948	0.987	ID											
E. amylovora	0.964	0.953	0.950	0.953	0.966	0.919	0.940	0.966	0.966	ID										
E. mallotivora	0.951	0.942	0.938	0.943	0.953	0.916	0.934	0.954	0.953	0.962	ID									
E. pyrifoliae	0.969	0.954	0.953	0.956	0.969	0.925	0.948	0.970	0.969	0.986	0.961	ID								
E. rhapontici	0.957	0.953	0.949	0.953	0.968	0.922	0.938	0.967	0.968	0.974	0.958	0.971	ID							
E. persicina	0.958	0.953	0.950	0.953	0.970	0.922	0.938	0.969	0.969	0.974	0.959	0.974	0.992	ID						
E. tracheiphila	0.941	0.938	0.936	0.938	0.939	0.906	0.933	0.946	0.943	0.940	0.937	0.944	0.937	0.938	ID					
E. psidii	0.953	0.951	0.948	0.948	0.958	0.920	0.943	0.960	0.958	0.958	0.966	0.961	0.958	0.959	0.951	ID				
E. toletana	0.946	0.962	0.956	0.961	0.962	0.919	0.943	0.967	0.967	0.961	0.949	0.962	0.971	0.973	0.941	0.954	ID			
P. stewartii	0.957	0.969	0.963	0.969	0.971	0.921	0.945	0.965	0.961	0.963	0.956	0.966	0.959	0.961	0.945	0.966	0.961	ID		
P. agglomerans	0.946	0.979	0.972	0.979	0.970	0.909	0.944	0.976	0.974	0.956	0.949	0.958	0.958	0.958	0.943	0.955	0.961	0.971	ID	
P. ananatis	0.952	0.979	0.974	0.979	0.968	0.912	0.951	0.974	0.970	0.957	0.949	0.959	0.954	0.956	0.948	0.957	0.965	0.978	0.982	ID

3.5 FIGURES

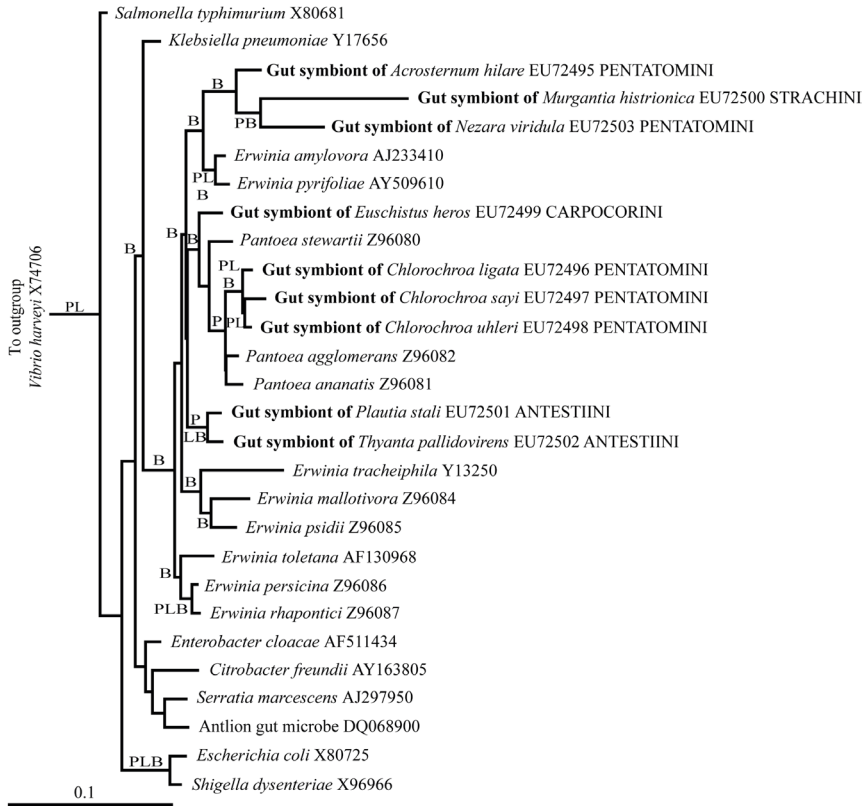


Figure 3.5.1. Phylogenetic placement of pentatomid gut symbionts (in bold) among closely related bacterial taxa. GS (pentatomid gut symbiont) precedes the species of insect host where the bacterium 16S rRNA gene sequence was obtained (in italics), which is followed by the accession number and pentatomid tribe the species belongs to (in capitals). Maximum likelihood tree is shown, but tree topology was similar with maximum parsimony and Bayesian searches. Capital letters represent branch support with >70% bootstrap replicates using maximum parsimony (P) (1000 bootstraps) and maximum likelihood (L) (250 bootstraps), and with >90% support with Bayesian analysis (B).

LITERATURE CITED

Abe, Y., K. Mishiro, and M. Takanashi. 1995. Symbiont of brown-winged green bug *Plautia stali* Scott. Jpn. J. Appl. Entomol. Zool. 39: 109-115.

Baumann, P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-suking insects. Annu Rev Microbiol 59: 55-89.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers, New York.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., Garrity, G.M. and J.M. Tiedje. 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 33: D294-D296.

DeSantis, T.Z., Hugenholtz, P., Keller, K., Brodie, E.L., Larsen, N., Piceno, Y.M., Phan, R., and G.L. Andersen. 2006. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res 34: W394-9.

Fukatsu, T., and N. Nikoh. 1998. Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). Appl Environ Microbiol 64: 3599-3606.

Fukatsu, T., and T. Hosokawa. 2002. Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. Appl Environ Microbiol 68: 389-396.

Hall, T.A. 1999. **BioEdit**: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp **41:** 95-98.

Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., and T. Fukatsu. 2006. Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. PLoS Biology 4: 1841-1851.

Huber, T., Faulkner, G., and P. Hugenholtz. 2004. Bellerophon; a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics **20:** 2317-2319.

Kikuchi, Y., Hosokawa, T., and T. Fukatsu. 2007. Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. Appl Environ Microbiol 73: 4308-4316.

Kikuchi, Y., Meng, X.Y., and T. Fukatsu. 2005. Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Hemiptera: Alydidae). Appl Environ Microbiol 71: 4035-4043.

Lockwood, J.A., and R.N. Story. 1986 Adaptive functions of nymphal aggregation in the southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae). Environ Entomol 15: 739-749.

Nylander, J.A.A. 2004. MrModeltest ver. 2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Posada, D., and K.A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Prado, S.S., Rubinoff, D., and R.P.P. Almeida. 2006. Vertical transmission of a pentatomid caeca-associated symbiont. Ann Entomol Soc Am 99: 577-585.

Ronquist, F., and P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Stackebrandt, E., and J. Ebers. 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiol. Today 33: 152-155.

Swofford, D.L. 2002. PAUP* Phylogenetic analysis using parsimony (*and other methods). Version 4. Computer program, version by D. L. Swofford. Sinauer, Sunderland, MA.

CHAPTER 4

COMPARATIVE DEMOGRAPHY OF GUT SYMBIOTIC AND APOSYMBIOTIC *Nezara viridula* (L.) (HEMIPTERA: PENTATOMIDAE)

Simone de Souza Prado

ABSTRACT: *Nezara viridula* (L.) is a highly polyphagous and cosmopolitan pentatomid stink bug. Despite its economic importance, aspects of its biology are poorly understood. *Nezara viridula* has one major bacterium associated with the last section of its midgut (V4), where the gastric caeca are located, which females provide to offspring by smearing it on the surface of eggs during oviposition. First instar nymphs hatch without the symbiont and acquire it orally by probing on the egg surface. We studied the impact of three temperatures and egg mass surface sterilization on *N. viridula*'s nymphal development rate and reproductive performance. Our results demonstrate that the symbiont's maintenance is affected by both temperature and egg mass surface sterilization. We detected the symbiont in 100%, 84% and 8.3% of the untreated control insects at 20, 25 and 30ºC, respectively, by using PCR. In insects originated from surface sterilized egg masses, the symbiont was never detected at 20 or 30ºC, and was detected in only 1 out of 21 insects at 25ºC. Nymphal mean development time decreased with increasing temperature, but there were no differences between the sterilized and control treatments. Sterilized insects at 20ºC lived longer than insects in any other treatment, but never laid eggs. Time to adult emergence was significantly slowed at 20ºC, but was not significantly different between sterilized (51.67±4.10 days) and control (38.25±6.61 days) treatments. Life table analysis of *N. viridula* female adults showed that net reproductive rate (R_0), intrinsic rate of increase (r), finite rate of increase (λ), and gross reproductive rate (GRR) were not significantly different among treatments except at 20ºC for the surface sterilized treatment. Mean generation time (T), however, was significantly longer at 20ºC (70.96±4.43) regardless of surface sterilization. Pre-oviposition and oviposition periods and number of eggs were not significantly different between the surface sterilized and control treatments at the three temperatures except at 20ºC for the surface sterilized treatment. Our results highlight the effect that temperature has on the maintenance of this symbiosis and its relationship with *N. viridula* host's development and reproductive life.

4.1 INTRODUCTION

The southern green stink bug *Nezara viridula* (L.) (Hemiptera: Pentatomidae) is a highly polyphagous and cosmopolitan insect, occurring on all continents, except Antarctica (Panizzi et al. 2000). Although unknown the most likely point of origin of *N. viridula* is the Ethiopian region of eastern Africa (see Todd 1989). In general, stink bugs have been recorded in many crops throughout most of the warmer regions of the world (Todd 1989), and are a major problem in soybean and other crops (Panizzi et al. 2000). Stink bugs attack host plants by inserting their piercing-sucking mouthparts into tissues and introducing digestive enzymes, which cause direct damage to the fruit and may eventually cause premature abscission of fruits. In addition, holes made by their stylets may allow the entrance of microorganisms that affect fruit quality (Jones and Caprio 1990, Panizzi et al. 2000).

Associations between microorganisms and insects are widespread in nature and can be obligate for the host, for the symbiont, for both, or for neither of them (Moran 2006). In general, the symbiotic bacteria associated with insects have been placed in two categories: primary or obligatory, and secondary or facultative (Baumann 2005). The best known symbiotic relationship is between the bacteria *Buchnera aphidicola* (Munson) and aphids. *Buchnera* is an obligatory symbiont of aphids that cannot live outside the host. *Buchnera* lives inside specialized cells called bacteriocytes, provides nutrients to the aphid, and is vertically (transovarially) transferred to the insect's offspring (Muson et al. 1991, Moran et al. 1993). It is been shown that use of antibiotics and heat treatments eliminate *Buchnera,* which in turn affects aphid development and fecundity (Douglas 1996, Montllor et al. 2002, Ohtaka and Ishikawa 1991, Russell and Moran 2006).

Secondary or facultative symbionts can be horizontally transmitted, creating the potential for multiple acquisitions of the symbionts by different species of insect. Facultative symbionts may confer upon the insect positive effects, such as heat stress tolerance (Montllor et al. 2002, Russell and Moran 2006), compensation for loss of *Buchnera* (Koga et al. 2003), resistance to parasitic wasps (Oliver et al. 2003), and resistance to pathogens (Scarbourough et al. 2005). Facultative symbionts can also have negative effects on growth, reproduction, and longevity of the host (Chen et al. 2000).

Buchner (1965) hypothesized that pentatomid stink bugs have an intimate relationship with caecum-associated bacteria. It has previously been shown that *N. viridula* and eight other species of pentatomids harbor bacterial symbionts in their posterior midgut (V4) and their phylogenetic placement showed they are polyphyletic (Prado and Almeida accepted). However, these nine different symbionts were placed together with *Erwinia* and *Pantoea* species, which are plant-associated bacteria.

Female stink bugs vertically transmit the bacteria to their offspring by smearing it on the top of the egg mass during oviposition, although specific details of this process are unknown. Aposymbiotic first instar stink bugs acquire the symbionts when they hatch and probe the surface of the egg (Abe et al. 1995, Buchner 1965, Prado et al. 2006). First instar nymphs remain aggregated on the surface of egg masses after hatching, a behavior previously hypothesized to provide protection against dessication (Lockwood and Story 1986) and potentially predators. In fact, it has been shown for *N. viridula* that this aggregation behavior results in faster insect development and reduced mortality (Lockwood and Story 1986). However, a similar behavior is also associated with symbiont acquisition (Hosokawa et al. 2008): plataspid stink bugs move more as first instars if symbiont capsules are removed from their egg masses. For pentatomids, such as *N. viridula*, a combination of protection from biotic and abiotic stresses in addition to acquisition of symbionts are plausible explanations for this behavior.

Prado et al. (2006) showed that growth and mortality of *N. viridula* at a controlled temperature (25±2ºC) was not affected by the absence of the primary symbiont. However, deprivation of mutualistic bacteria in insects of other families in the infraorder pentatomorpha resulted in slowed growth and/or higher nymphal mortality (Abe et al. 1995, Fukatsu and Hosokawa 2002, Hosokawa et al. 2006).

Numerous studies on demography of insects provide important information on factors affecting population growth, but this approach has not been previously used to study the role of bacterial symbionts in insect host populations. For example, life table construction has been used as a demographic measure to compare efficiency of insecticides on stink bugs (Zanuncio et al. 2005), to compare different rearing techniques and diets for stink bugs (Fortes et al. 2006), and to quantify life history traits at different temperatures (Legaspi 2004). Previous work showed that temperature may interfere with insect reproduction and life history through elimination of the insect's primary symbionts (e.g. Ohtaka and Ishikawa 1991). We hypothesized that different temperatures might affect the primary symbiont of *N. viridula*, decrease reproductive performance and subsequent development. In this study, using demographic studies we compare various demographic parameters between symbiotic and aposymbiotic *N. viridula* at three different temperatures.

4.2 MATERIALS AND METHODS

4.2.1 Insect colonies

Nezara viridula adults were collected from multiple locations and host plants on the island of Hawaii and reared in screen cages (28x52x54cm) at the USDA-ARS laboratory in Hilo (HI) at 25±2ºC and a photoperiod of 14:10 (L:D). *Nezara viridula*

was fed fresh green beans, cabbage and non-salted roasted peanuts. Adults mate randomly and wild caught insects were added to the colony every generation. To start the experiments, we collected 200 eggs laid on the same day, and randomly assigned them to two treatments: surface sterilized eggs or unsterilized controls. In the surface sterilized treatment, egg masses were treated with 10% bleach for 5 minutes (Prado et al. 2006). The controls were rinsed with water. After treatment, eggs were transferred to 3.8-liter plastic tubs (Rubbermaid, Wooster, OH) with screened lids and supplied with beans, cabbage and peanuts when they hatched. Containers with eggs were placed in growth chambers set at one of three temperatures: 20, 25 and 30ºC. There were four replicates of each temperature-sterilization treatment combination in a factorial design. Food was replaced every other day or as necessary. Newly metamorphosed adults were confined in a screen cage (30.4x30.4x30.4 cm) separate from the nymphs and allowed to mate. Paper towels cut in 2 inch strips were taped on the walls as an oviposition substrate and fresh food was added as described above. Once a week, all of the adult cages were checked, removing any egg masses present; adults and nymphs were censused daily to record development and mortality.

4.2.2 Symbiont detection

We collected fifth instar (final nymphal stage) and adult *N. viridula* from surface-sterilized and control treatments at all temperatures and stored them at -20ºC for symbiont detection. A total of 56 insects (5th instars and adults) in the control treatment and 26 insects in the surface sterilized treatment were tested. We dissected the insects and collected the V4 section of the midgut. We extracted DNA from insect tissue and used PCR to detect the bacterium as previously described (Prado et al. 2006).

4.2.3 Demography parameters

We determined the median development time (MDT) by calculating the cumulative proportion of the insect's population that passed through a given stage on a given day. The estimates provided by this method should be relatively robust to influences of mortality. These cumulative proportions were then arcsin square root transformed to linearize results with respect to time. We then fit separate linear regressions for each stage, and used the regression to calculate the number days required for 50% of the population to pass beyond stage j (=MDT j). We used these methods (Peterson and Painting 1990) to calculate MDT from the day that the insects hatched through the final nymphal stage - 1st instar, 2nd instar, 3rd instar, 4th instar and 5th instar- at all temperatures.

Both stage-specific and cumulative survivorships were calculated for each replicate separately. We also measured developmental time of nymphal stages as time (days) within each stadium. In addition we calculated several life table parameters: net reproductive rate (R_0), mean generation time (T), intrinsic rate of increase (r), finite rate of increase (λ) and gross reproductive rate (GRR), adult emergence and oviposition parameters (pre-oviposition period, oviposition period, total number of eggs) as described by Carey (1993a). For these calculations, we assumed a sex ratio of 50%. Finally, life expectancies were calculated considering longevity (T_x - days lived beyond age x) and survival rate (l_x - survival from age zero to the beginning of age x) by the approximated method (Southwood 1978): $e_x=T_x/l_x$, described by Carey (1993b).

4.2.4 Statistical analyses

We used the software R v. 2.6.1 to perform the statistical analyses (R Development Core Team 2007). The total proportion of insects positive for symbiont detection at 20, 25 and 30ºC was compared using a logistic regression, with sterilization treatment as a fixed effect and temperature a covariate. For MDT statistical analyses, we used a linear mixed effects model with development stage as a random, repeated measure (Crawley 2005). This method allowed us to compare differences in MDT among temperature and sterilization treatments, but not among stages. MDT was used as log transformed MDT to meet the test assumptions. Significant main effects of treatments were followed up with pairwise comparisons among sterilization treatments and among temperatures. The effect of temperature on life table parameters (R_0, T, r, λ, and GRR), adult emergence (day that the first adult appeared), and oviposition parameters (pre-oviposition period, oviposition period, total number of eggs) were analyzed using separate one-way ANOVAs with five treatment levels (20ºC-Control, 25ºC-Control, 30ºC-Control, 25ºC-SS, and 30ºC-SS). This approach was used because insect survival was so low in the 20ºC SS treatment that the parameters could not be estimated for any of the four replicates. The exception was for adult emergence, for which a 2x3 factorial ANOVA was used. We log transformed GRR to meet test assumptions. Significant main effects were tested by two tests: contrasts between control and surface sterilized, and contrasts between 25ºC and 30ºC degrees. Pairwise t-tests were used to compare means among temperatures within each sterilization treatment with Bonferroni adjustments to significance values to account for multiple comparisons.

4.3 RESULTS

4.3.1 Symbiont detection

We detected *N. viridula*'s symbiont in 100% of the insects at 20ºC, in 84% of the insects at 25ºC and in 8.3% of the insects at 30ºC in the control treatment (Figure 4.6.1). In the surface sterilized treatment only one insect out of eleven tested was positive at 25ºC. We used a logistic regression to evaluate the effect of temperature on symbiont maintenance in surface-sterilized and control treatments. Overall, there was a significant effect of temperature ($z = 2.468$; $p = 0.0136$) and of the interaction between temperature and treatment ($z = -1.983$; $p = 0.0474$). Thus, temperature increments seem to affect symbiont maintenance. Pairwise comparisons between sterilized and non-sterilized treatments showed statistical differences at 20ºC and 25ºC, but not at 30ºC (Figure 4.6.1).

4.3.2 Demography parameters

4.3.2.1 Median development time (MDT)

The MDT for the surface sterilized treatment from the day that the insects hatched until the final nymphal stage (5th instar) was 62.73±3.59, 39.70±1.09, and 26.54±1.07 days at 20ºC, 25ºC and 30ºC respectively (Figure 4.6.2). For the control treatment, MDT was 47.98±7.92, 34.87±2.01, and 26.07±1.56 days at 20ºC, 25ºC and 30ºC respectively. Results showed a significant effect of temperature ($z = -4.984$, $p = 0.0001$), but we found no significant effect of treatment. There was a tendency for control insects to develop faster than surface sterilized treatment at 20ºC and 25ºC, but not at 30ºC (Figure 4.6.2). The interaction between temperature and treatment was also not statistically different. Pairwise comparisons showed significant differences between 20ºC and 25ºC ($t = -2.236$, d.f. = 11, $p = 0.047$) and between 30ºC and 20ºC ($t = -5.413$, d.f. = 12, $p < 0.0001$), but not between 30ºC and 25ºC ($t = -3.216$, d.f. = 11, $p = 0.082$).

4.3.2.2 Biological parameters

Summarized life table parameters are presented in Table 4.5.1. Statistical analyses comparing the effect of temperature on life table parameters between surface sterilized and control treatments showed that temperature did not have an effect on R_0 values; R_0 was highest at 25ºC. Mean generation time was significantly different for the temperature treatments. Pairwise comparisons among all three control temperatures showed significantly longer generation time at 20ºC (70.96±4.43)

than 25ºC (46.59±5.54, $p = 0.0045$) and 30ºC (50.67±4.47, $p = 0.0131$). Comparisons between the surface sterilized treatment and control treatment and between 25ºC and 30ºC did not show significant differences. The life table parameters r, λ, and *GRR* were not significantly affected by temperature or sterilization treatment. Time of adult emergence was significantly different between the temperature treatments; pairwise comparisons showed a significantly longer time to adult development ($t = 3.278$, $p = 0.0074$) at 20ºC, but did not show any significant difference between surface sterilized (51.67±4.10) and control (38.25±6.61) treatments (Table 4.5.2). The parameters pre-oviposition period, oviposition period, and number of eggs laid were not significantly affected by temperature or sterilization treatment (Table 4.5.2). Except that at 20ºC of the surface sterilized treatment we could not calculate the life table parameters because of the high mortality of the nymphs. In addition, adults emerged in only two replicates, and never laid eggs for the surface sterilized at 20ºC treatment. Females laid more eggs at 25ºC in the control (243.50±63.78) and surface sterilized (174±107) treatments than at 20ºC and 30ºC, but the means were not significantly different.

The effect of temperature on *N. viridula*'s life expectancy is presented in Figure 4.6.3. The average life expectancy data clearly showed survivorship and longevity differences among the treatments (not analyzed statistically). The average life expectancy of insects at 30 ºC in both the surface sterilized and control treatments and insects of the surface sterilized treatment at 25ºC were low and similar. However, the control treatment at 25ºC showed a higher expectation of life and the insects survived for longer period (252 days, laying eggs during 12.50±4.84 days) than the sterilized treatment (90 days laying eggs during 7 days). At 20ºC adults of the surface sterilized treatment had the highest life expectancy and the longest longevity around 261 days, but they never laid eggs. At 20ºC of the control treatment, *N. viridula*'s showed a tendency of higher life expectancy and longevity than insects reared at 30ºC, but lower than the insects reared at 25ºC of the control treatment.

4.4 DISCUSSION

The effects of temperature and egg mass surface sterilization on *N. viridula*'s development and maintenance of a gut symbiont were studied. In general, our results demonstrate that the symbiont's maintenance in *N. viridula* was directly affected by surface sterilization of the egg masses and temperature. At 30ºC the symbiont was detected in only 2 of the 24 (8.3%) insects in the control treatment. As previously demonstrated, heat can negatively impact the population of the aphid primary symbiont, *Buchnera*, causing host infertility (Montllor et al. 2002). Subjecting aphids to high temperatures when immature causes them to lose their obligatory symbiont, which may be compensated by the presence of facultative

symbionts (Montllor et al. 2002, Koga et al. 2003, Russell and Moran 2006). In the case of *N. viridula* it is unknown if any mechanism exists to compensate for the lack of its symbiont at high temperatures. Further studies are necessary to clarify the relationships observed here and to determine the impact of temperature on this association under field conditions.

As in previous reports (Harris and Todd 1981, Vivan and Panizzi 2005), our results indicate that *N. viridula*'s nymphal developmental time was reduced with increasing temperature. Our experiments were not able to clearly decouple the proportional effect of high temperature on the insect host alone and loss of the gut symbiont. Surface sterilization did not statistically affect nymphal development time, but there was a tendency toward slower nymphal development time in insects without symbionts (surface sterilized treatment). *N. viridula* that originated from surface sterilized egg masses in the 20ºC treatment had the longest nymphal developmental time and the longest mean generation time (*T*). In addition, the insects in the surface sterilized treatment at 20ºC had the highest values of life expectancy, the longest time to adult emergence and the greatest longevity, yet adults (n = 13) never laid eggs. These results indicate that the symbiont has an important role in determining development time, survivorship, longevity, and generation time of *N. viridula*. Furthermore, it suggests that the symbiont provides the host with nutritional factors that become more important at lower temperatures. The median development time between surface sterilized and control insects at 30ºC was statistically similar, consistent with the fact that *N. viridula* lost its gut symbionts at higher temperatures. Previously, Panizzi (2002) reported that *N viridula* is expanding in the tropic regions towards warmers areas. If this trend is true, it may have an important implication for *N. viridula* life history, since an upper temperature threshold may exist beyond which *N. viridula* populations relationship with their symbiont may be affected, potentially reducing insect fitness. These considerations need to be investigated given current climate change forecasts (Walther et al. 2002).

Numerous strategies for symbiont transfer by females to their offspring have been described for the suborder Heteroptera (Buchner 1965, Durvasula et al. 1999, Fukatsu and Hosokawa 2002, Hosokawa et al. 2006, Prado et al. 2006). In the family Pentatomidae, the smearing of the symbiont on the top of the egg masses by the female while ovipositing is a unique mode of transmission. This distinctive mechanism allows environmental factors to interfere the symbiosis and influence in its polyphyletic evolutionary interaction between insect-symbiont, as in each new generation the symbiont is exposed to constant environmental changes and this too may be negatively affected by increased temperature.

Several authors studied nymphal duration of *N. viridula* (Jones and Brewer 1987, Noda and Kamano 2002, Panizzi and Saraiva 1993), but all of them they

ignored the first instar duration, only counting from 2nd instar to adults. Here, we showed that entire nymphal developmental time for *N. viridula* was 47.98±7.92, 34.87±2.01, and 26.07±1.56 days at 20ºC, 25ºC and 30ºC respectively, consistent with previous studies (Ali and Eweiss 1977, Harris and Todd 1980). Similar results for the biological parameters observed among temperatures in this study may be due to a fitness cost incurred by the insect owing to the loss of the symbiont at 30ºC. In addition, at lower temperatures the symbiont's absence had a direct effect on the host by inhibiting reproduction. Additionally, *N. viridula*'s diet used in this research should be considered, although it was consistent across all treatments. We used green beans that are a good source of carbohydrates and protein and it has been shown that together with peanuts are adequate for *N. viridula*'s development (Todd 1989). Such an unusually rich diet may have masked differences in fitness among the treatments tested here.

At 25ºC *N. viridula*'s population tended to have higher R_0 and lower *T* in both surface sterilized and control treatments. Although *GRR* tended to be lower at the sterilized treatment, R_0 provides a more powerful interpretation of the data value because it incorporates mortality rate during nymphal and adult stages of the organisms (Force and Messenger 1964); however instantaneous rate of increase (*r*) is more commonly used to compare populations of organisms under different conditions because it relates net reproductive rate with generation time (Carey 1993a). For example at 30ºC, the surface sterilized treatment presented a negative value of *r* meaning that the population was not increasing, due to low values of R_0 and *T*. At 25ºC although not significant, the total number of eggs laid by *N. viridula*'s female was higher 30 ºC in both surface sterilized and control treatments. These findings reinforce results from previous work, that *N. viridula*'s fitness appeared not to be affected when deprived of its dominant symbiont by surface sterilization of egg masses, suggesting the insect's low level of symbiont dependency at the optimal temperature, 25ºC (Prado et al. 2006).

This research highlights the importance of the use of demographic studies to demonstrate the effects of bacterial symbionts in the development of insects. Interesting, high temperature cleans off the gut-associated symbionts from *N. viridula* and fastened its development; however at cooler temperatures no reproduction was recorded when the symbiont is removed. These results suggest that absence or presence of symbionts and/or its interaction with abiotic factors must be taking into account in order to understand the entire ecology of natural populations of *N. viridula* and this could have implications in the pentatomid pest management.

4.5 TABLES

Table 4.5.1. Effect of temperature and surface sterilization on *Nezara viridula*'s demography parameters.

Factors	20˚C		25˚C		30˚C	
	Control (n[1])	SS (n)	Control (n)	SS (n)	Control (n)	SS (n)
R_0 (♀/♂)[2]	2.13±0.95A (4)	-[3]	2.80±0.83A (4)	2.25±1.58A (2)	1.17±0.53A (4)	1.43±0.85A (2)
T (days)[4]	70.96±4.43A (4)	-	46.59±5.54B (4)	43.00±8.00B (2)	50.67±4.47B (4)	40.68±5.68B (2)
r (♀/♀/day)[5]	0.01±0.01A (4)	-	0.02±0.01A (4)	0.02±0.02A (2)	-0.01±0.01A (4)	0.001±0.017A (2)
λ (♀/♀/day)[6]	1.01±0.01A (4)	-	1.02±0.01A (4)	1.02±0.02A (2)	0.99±0.01A (4)	1.001±0.017A (2)
GRR[7]	75.85±50.90A (4)	-	58.35±11.42A (4)	18.24±8.56A (2)	55.85±41.80A (4)	29.34±24.06A (2)

[1] Total number of replicates.

[2] Net reproductive rate – $R_0=\sum l_x m_x$; Means followed by the same capitalized letters in the same line are not significantly different ($F = 0.5902$, d.f. = 4, $p = 0.677$).

[3] – No data for this treatment.

[4] Mean generation time – $T=(\sum x\, l_x m_x)/R_0$; Means followed by different capitalized letters in the same line are significantly different ($F = 5.2448$, d.f. = 4, $p = 0.013$).

[5] Instantaneous rate of increase – $r=ln(R_0)/T$; Means followed by the same capitalized letters in the same line are not significantly different ($F = 0.5966$, d.f. = 4, $p = 0.673$).

[6] Finite rate of increase – $\lambda=exp\,(r)$; Means followed by the same capitalized letters in the same line are not significantly different ($F = 0.6029$, d.f. = 4, $p = 0.669$).

[7] Gross reproductive rate – $GRR=\sum m_x$; Means followed by the same capitalized letters in the same line are not significantly different ($F = 0.5262$, d.f. = 4, $p = 0.719$).

Table 4.5.2. Effect of temperature and surface sterilization on adult emergence and reproductive performance of *Nezara viridula*'s females.

Factors	20˚C		25˚C		30˚C	
	Control (n[1])	SS (n)	Control (n)	SS (n)	Control (n)	SS (n)
Adult emergence[2]	38.25±6.61A (4)	51±3.5A (2)	27.75±1.25B (4)	30.00B (2)	20.50±1.38B (4)	20.67±2.73B (3)
Pre-oviposition period[3]	28.25±6.80A (4)	-	14.75±3.66A (4)	13.00±8.00A (2)	29.00±3.39A (4)	17.00±1.00A (2)
Oviposition period[4]	13.75±6.75A (4)	-	12.50±4.84A (4)	7.00A (2)	10.25±3.25A (4)	12.00±5.00A (2)
Number of eggs[5]	170.50±65.78A (4)	-	243.50±63.78A (4)	174.00±107.00A (2)	118.00±45.47A (4)	107.00±49.00A (2)

[1] Total number of replicates.

[2] Means followed by different capitalized letters in the same line are significantly different ($F = 6.6604$, d.f. = 5, $p = 0.003$).

[3] Means followed by the same capitalized letters in the same line are not significantly different ($F = 2.1628$; d.f. = 4, $p = 0.141$).

[4] Means followed by the same capitalized letters in the same line are not significantly different ($F = 0.1957$; d.f. = 4, $p = 0.935$).

[5] Total number of eggs laid during the entire female lifetime; Means followed by the same capitalized letters in the same line are not significantly different ($F = 0.7238$, d.f. = 4, $p = 0.594$).

4.6 FIGURES

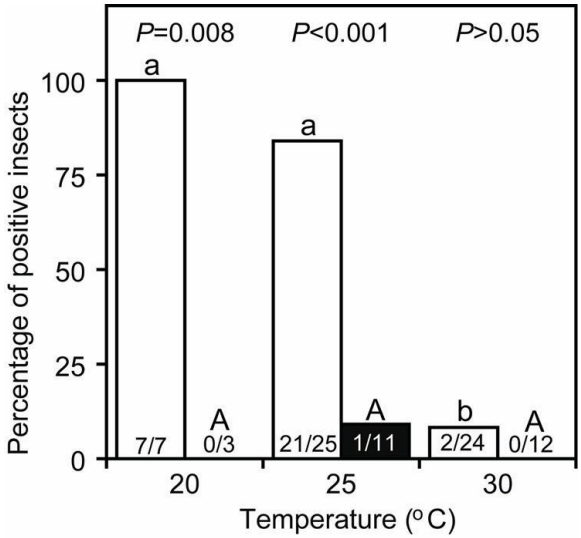


Figure 4.6.1. Percentage of symbiont positive insects at 20, 25 and 30ºC. Columns with the same capital letters and the same lower case letters do not differ significantly (p<0.05), χ^2 test. Outcomes of statistical analyses between treatments in each temperature are represented by p-values above the bars. White columns represent control treatment and black columns represent the surface sterilized treatment.

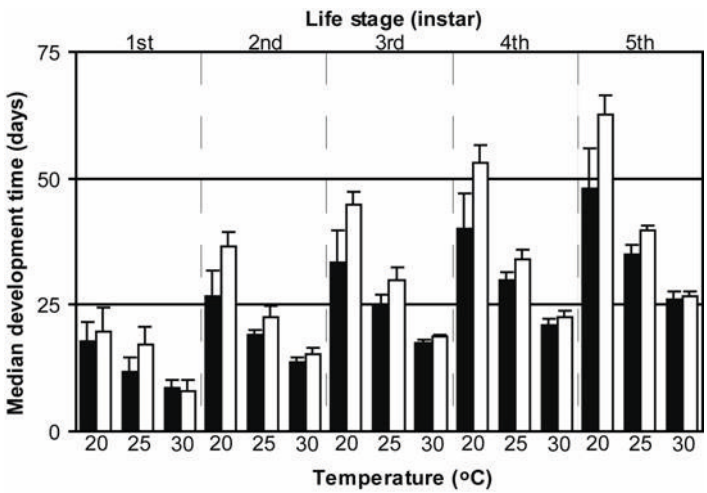


Figure 4.6.2. Mean nymphal development time (±SE) in the 5 life stage of *Nezara viridula* at 20, 25 and 30ºC. Black columns represent control treatment and white columns represent surface sterilized treatment.

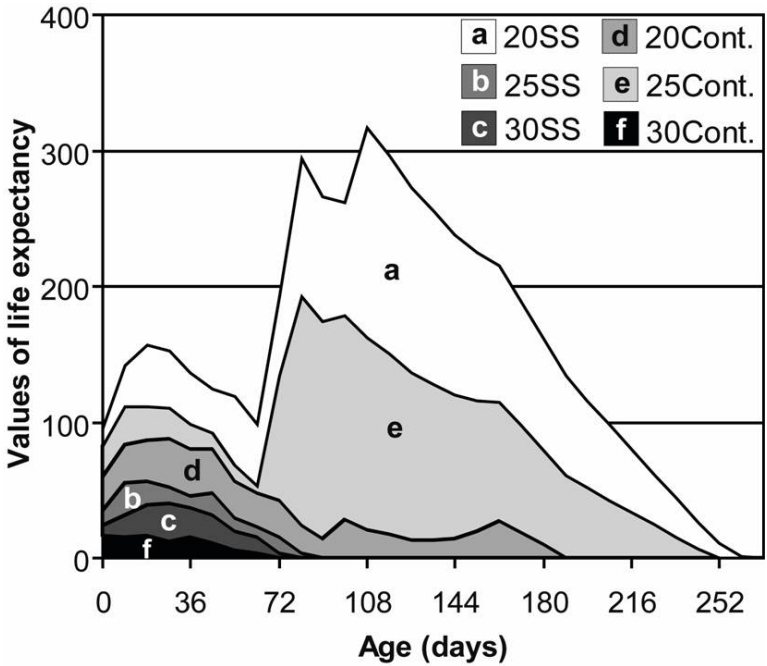


Figure 4.6.3. Mean values of life expectancy for on surface sterilized (SS) treatments (a, b and c) and on control (Cont.) treatments (d, e and f) at 20, 25 and 30ºC.

LITERATURE CITED

Abe, Y., K. Mishiro, and M. Takanashi. 1995. Symbiont of brown-winged green bug *Plautia stali* Scott. Jpn. J. Appl. Entomol. Zool. 39: 109-115.

Alis, M., and M.A. Eweiss. 1977. Photoperiodic and temperature effects on rate of development and diapause in the green stink bug, *Nezara viridula* L. (Heteroptera: Pentatomidae). Z. Ang. Ent. 84: 256-264.

Baumann, P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-suking insects. Annu. Rev. Microbiol. 59: 55-89.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers, New York.

Carey, J.R. 1993a. Applied demography for biologists with special emphasis on insects. Oxford University Press, Oxford, England.

Carey, J.R. 1993b. Longevity – The biology and demography of life span. Princeton University Press, New Jersey.

Chen, D.Q., Montllor, C.B., and A.H. Purcell. 2000. Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfafa aphid, *A. kondoi*. Entomol. Experim. Appl. 95: 315-323.

Crawley, M.J. 2005. Statistics: An introduction using R. John Wiley & Sons, England.

Douglas, A. E. 1996. Reproductive failure and the amino acid pools in pea aphids (*Acyrthosiphon pisum*) lacking symbiotic bacteria. J. Insect Physiol. 42: 247-255.

Durvasula, R.V., Kroger, A., Goodwin, M., Panackal, A., Kruglov, O., Taneja, J., Gumbs, A., Richards, F.F., Beard, C.B. and C. Cordon-Rosales. 1999. Strategy for introduction of foreign genes into field populations of chagas disease vectors. Ann. Entomol. Soc. Amer. 92: 937-943.

Force, D.C., and P.S. Messenger. 1964. Fecundity, reproductive rates and innate capacity for increase of three parasites of *Therioaphis maculate* (Buckton). Ecol. 45: 706-715.

Fortes, P., Magro, S.R. Panizzi, A.R. and J.R.P. Parra. 2006. Development of a dry artificial diet for *Nezara viridula* (L.) and *Euschistus heros* (Fabricius) (Heteroptera: Pentatomidae). Neotrop. Entomol. 35: 567-572.

Fukatsu, T., and T. Hosokawa. 2002. Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. Appl. Environ. Microbiol. 68: 389-396.

Harris, V.E. and J.W. Todd. 1980. Duration of immature stages of the southern green stink bug, *Nezara viridula* (L.), with a comparative review of previous studies. J. Georgia Entomol. Soc. 15: 114-124.

Harris, V.E. and J.W. Todd. 1981. Rearing the southern green stink bug, *Nezara viridula*, with relevant aspects of its biology. J. Georgia Entomol. Soc. 16: 203-210.

Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., and T. Fukatsu. 2006. Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. PLoS Biology 4: 1841-1851.

Hosokawa, T., Kikuchi, Y., Shimada, M., and T. Fukatsu. 2008. Symbiont acquisition alters behavior of stinkbug nymphs. Biol. Lett. 4: 45-48.

Jones, V.P., and L.C. Caprio. 1990. Biology and control of insect pests attacking macadamia nuts in Hawaii. Proc. Hawaii Macadamia Nut Assoc. 30: 24-36.

Jones, W.A., and F.D. Brewer. 1987. Suitability of various host plant seeds and artificial diets for rearing *Nezara viridula* (L.). J. Agri. Entomol. 4: 223-232.

Koga, R., Tsuchida, T., and T. Fukatsu. 2003. Changing partners in an obligate symbiosis: a facultative endosymbiont *Buchnera* in an aphid. Proc. R. Soc. Lond. B. 270: 2543-2550.

Legaspi, J.C. 2004. Life history of *Podisus maculiventris* (Heteroptera: Pentatomidae) adult females under different constant temperatures. Environ. Entomol. 33: 1200-1206.

Lockwood, J.A., and R.N. Story. 1986. Adaptive functions of nymphal aggregation in the southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae). Environ. Entomol. 15: 739-749.

Montllor, C.B., Maxmen, A., and A.H. Purcell. 2002. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecol. Entomol. 27: 189-195.

Moran, N.A. 2006. Symbiosis. Curr. Biol. 16: R866-R871.

Moran, N.A., Munson, M.A., Baumann, P., and H. Ishikawa. 1993. Molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. Proc. R. Soc. Lond. B. 253: 167-171.

Munson, M.A., Baumann, P., Clark, M.A., Baumann, L., Moran, N.A., Voegtlin, D.J., and B.C. Campbell. 1991. Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. J. Bact.173: 6321-6324.

Noda, T., and S. Kamano. 2002. Artificial rearing of *Nezara viridula* (L.) and *N. antennata* Scott (Heteroptera: Pentatomidae) with semi-solid meridic diets. Appl. Entomol. Zool. 37: 43-50.

Ohtaka, C., and H. Ishikawa. 1991. Effects of heat treatment on the symbiotic system of an aphid mycetocyte. Symbiosis 11: 19-30.

Oliver, K.M., Russell, J.A., Moran, N.A., and M.S. Hunter. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc. Nat. Acad. Sci. Unit. Sta. Amer. 100: 1803-1807.

Panizzi, A.R. 2002. Stink bugs on soybean in northeastern Brazil and a new record on the southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae). Neotrop. Entomol. 31: 331-332.

Panizzi, A.R., and S.I. Saraiva. 1993. Performance of nymphal and adult southern green stink bug on an overwintering host plant and impact of nymph to adult food-switch. Entomol. Exp. Appl. 68: 109-115.

Panizzi, A.R., McPherson, J.E., James, D.G., Javahery, M., and R.M. McPherson. 2000. Economic importance of stink bug (Pentatomidae). In Schaefer C.W. and A.R. Panizzi [eds.], Heteroptera of economic importance, CRC, Boca Raton, Florida.

Peterson, W.T., and S.J. Painting. 1990. Developmental rates of the copepods *Calanus australis* and *Calanoides carinatus* in the laboratory, with discussion of methods used for calculation of development time. J. Plankton Res. 12: 283-293.

Prado, S.S., and R.P.P., Almeida. 2008. Phylogenetic placement of pentatomid stink bug gut symbionts. Curr. Microbil. Accepted.

Prado, S.S., Rubinoff, D., and R.P.P. Almeida. 2006. Vertical transmission of a pentatomid caeca-associated symbiont. Ann. Entomol. Soc. Am. 99: 577-585.

R Development Core Team 2006 R: a language and environment for statistical computing. Vienna, Austria: R Foundation for statistical computing. See http://www.R-project.org

Russell J.A., and N.A. Moran. 2006. Cost and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proc. R. Soc. Lond. B. 273: 603-610.

Scarbourough, C.L., Ferrari, J., and H.C. Godfray. 2005. Aphid protected from pathogen by endosymbiont. Science. 310: 1781.

Southwood, T.R.E. 1978. Ecological methods with particular reference to the study of insect populations, 2nd ed. Chapman & Hall, London, United Kingdom.

Todd, J.W. 1989. Ecology and behavior of *Nezara viridula*. Ann. Rev. Entomol. 34: 273-292.

Vivan L.M., and A.R. Panizzi. 2005. Nymphal and adult performance of genetically determined types of *Nezara viridula* (L.) (Heteroptera: Pentatomidae), under different temperature and photoperiodic conditions. Neotrop. Entomol. 34: 911-915.

Walther, G.-R., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T.J.C., Fromentin, J.-M., Hoegh-Guildberg, O., and F. Bairlein. 2002. Ecological responses to recent climate change. Nature. 416: 389–395.

Zanuncio, T.V., Zanuncio, J.C., Serrao, J.E., Medeiros, R.S., Pinon, T.B.M., and C.A.Z. Sediyama. 2005. Fertility and life expectancy of the predator *Supputius cincticeps* (Heteroptera: Pentatomidae) exposed to sublethal doses of permethrin. Biol. Res. 38: 31-39.

CHAPTER 5

ROLE OF GUT SYMBIOTIC BACTERIA IN THE DEVELOPMENT OF *Acrosternum hilare* AND *Murgantia histrionica* STINK BUGS (HEMIPTERA: PENTATOMIDAE)

Simone de Souza Prado

ABSTRACT: The green stink bug, *Acrosternum hilare* (Say) and the harlequin bug, *Murgantia histrionica* (Hahn) are pests of many economically important crops. Although both species have been extensively studied, their relationship with symbiotic gut bacteria remains unknown. Their potential relationship with endosymbionts may be important, as other pentatomid species harbor vertically transmitted gut bacteria that play an important role in the biology of the host insects. In this work we report the effect of gut symbiotic bacteria on the development and fitness of *A. hilare* and *M. histrionica* by comparing control insects with individuals that hatched from surface sterilized egg masses. We studied the life history of *A. hilare* and *M. histrionica* in laboratory conditions at 23±2ºC and at photoperiod of 16:8 [L:D]. We censused the insects after hatch and calculated nymphal mortality, development time and demographic parameters. We also evaluated egg masses, nymphs and adults of both species with diagnostic PCR primers for presence of specific gut symbiotic bacteria at control and surface sterilized treatments. Our results determined that egg mass surface sterilization eliminates or reduces the frequency of this bacteria-insect gut symbiosis in both species. *Acrosternum hilare*'s development time, survivorship and demographic parameters were negatively affected by surface sterilization of egg masses. Conversely, *M. histrionica*'s survivorship was slightly increased by clearing its symbiont infection during the first generation; however, mean generation time was significantly longer. Our data suggest that egg smearing is a commonly used mechanism for vertical transmission of pentatomid gut associated symbionts. Our data also suggest that the degree of mutualism of this association for pentatomid species is variable, given the fact that *A. hilare* requires its symbiont for adequate development, while *M. histrionica*'s symbiont may not be significant in development of the host insect.

5.1 INTRODUCTION

Microbial symbionts may be biologically important to their hosts, and may influence the host's evolution (Baumann 2005). Insects are often hosts to symbionts, and have remarkably diverse symbiotic associations with microorganisms and have been of interest to biologists because of this diversity ranging from obligate benefic symbionts to reproductive malefic parasites of mutual importance (Buchner 1965). Symbiotic associations among insects of the order Hemiptera and their associated bacteria are present in its three suborders: Sternorrhyncha (e.g. aphids, mealybugs, whiteflies, psyllids, etc.), Auchenorryhyncha (e.g. spittlebugs, planthopers, leafhoppers, treehoppers, etc.), and Heteroptera (true bugs) (Baumann 2005, Fukatsu and Hosokawa 2002, Hosokawa et al. 2006, Prado et al. 2006). The suborders Sternorrhyncha and Auchenorryhyncha include insects of economic importance that cause direct plant damage or transmit plant pathogens. Heteroptera also includes economically important insects that feed on plants and blood as well as on other insects (Panizzi et al. 2000).

Extensive work on symbiotic associations of Sternorrhyncha insects has been performed (Baumann 2005). The best explored symbiotic relationship is that of the bacterium *Buchnera aphidicola* and the aphid *Acyrthosiphon pisum* (Harris) (Muson et al. 1991, Moran et al. 1993, Shigenobu et al. 2000). In addition, mealybugs, whiteflies, and psyllids also harbor their own obligatory endosymbionts that are vertically (maternally) transmitted to offspring (Baumann 2005). These endosymbiotic relationships are believed to be the result of a single ancestral infection caused by vertical transmission, and have resulted in the reduction of the bacteria's genome size (Baumann 2005, Moran and Mira 2001, Shigenobu et al. 2000, Tamas et al. 2002). Eventually, these bacteria became confined inside specialized cells of the hosts, called bacteriocytes, and the bacteria lack of various essential genes that previously made life outside the host impossible (Buchner 1965, Baumann 2005, Douglas 1996, Shigenobu et al. 2000, Tamas et al. 2002).

In Heteroptera, some symbionts are found in the gut lumen (Reduviidae) or in the gastric caeca (Pentatomidae, Plataspidae, Alydidae, etc.) and are not transovarially transmitted (Buchner 1965, Durvasula et al. 1999, Fukatsu and Hosokawa 2002, Prado et al. 2006). In Cimicomorpha (Reduviidae), for example, the vector of the Chaga's disease pathogen *Rhodnius prolixus* (Stal), acquires a bacterial symbiont, *Rhodococcus rhodnii,* soon after the first instar hatches by probing on adult's fecal pellets (Durvasula et al. 1999, Lawyer and Perkins 2000). These bacterial symbionts are orally transmitted through 'contamination' of materials associated with the egg masses, or fecal pellets (Buchner 1965, Durvasula et al. 1999, Fukatsu and Hosokawa 2002, Hosokawa et al. 2006, Prado et al. 2006). This unique model of transmission may require higher genome stability or prevent loss of genes required for living

outside the host by the symbionts and it includes an extra challenge to the symbiont, which must survive outside its host in a constantly changing environment (Prado et al. 2006, Prado et al. unpublished).

The Japanese stink bug, *Megacopta punctatissima* (Montandon) (Pentatomorpha, family Plataspidae) also harbors a bacterial symbiont '*Candidatus* Ishikawaella capsulata' in their gastric caeca (Fukatsu and Hosokawa 2002, Hosokawa et al. 2006). *Megacopta punctatissima* females attach small capsules filled with symbionts to their egg masses. After hatching, the nymphs immediately probe on these capsules and acquire the symbionts. It has been shown that *M. punctatissima* and its symbiont are strictly cospeciated and the symbiont's removal causes retarded growth, mortality and sterility of the insects (Fukatsu and Hosokawa 2002, Hosokawa et al. 2006, Hosokawa et al. 2007). Conversely, other work with *Riptortus clavatus* (Thunberg) and *Leptocorisa chinensis* (Dallas) (Pentatomorpha, family Alydidae) demonstrated that their dominant symbionts are not vertically transmitted by females, but are environmentally acquired each generation by first instar nymphs from soils and are not monophyletic (Kikuchi et al. 2005, 2007).

Insects of the family Pentatomidae (Pentatomorpha) harbor a dominant bacterium in the last section of its midgut, where the gastric caeca is located (V4) (Abe et al. 1995, Buchner 1965, Hirose et al. 2006, Prado et al. 2006, Prado and Almeida 2008, Prado et al. unpublished). Symbionts are smeared on the top of the egg masses by females and newly hatched nymphs probe on the egg surface to acquire them (Abe et al. 1995, Buchner 1965, Prado et al. 2006). Despite their potential importance, symbiotic associations in pentatomid insects have not been subjected to detailed study. Abe et al. (1995) showed that *Plautia stali* Scott died before turn adults because of the absence of the symbiont when their egg masses are surface sterilized. *Nezara viridula*'s (L.) development and biology was not clearly affected by the removal of their symbionts at 25ºC (Prado et al. 2006). In addition, high temperatures have been shown to negatively impact retention of symbionts by *N. viridula*. (Prado et al. unpublished). Phylogenetic analyses of the symbionts of nine different species of stink bugs showed that they are polyphyletic, and they are placed together with *Erwinia* and *Pantoea*, which are plant pathogens (Prado and Almeida 2008). Although stink bug gut symbionts are polyphyletic, suggesting that they may be replaced with taxonomically similar bacteria over evolutionary time and that different species of insects may have a different level of association, more work needs to be done in the attempt to explore the role symbionts have on insect life history. In this study we investigated the impact that vertically transmitted symbionts have on development and reproduction of the green stink bug, *Acrosternum hilare* and the harlequin bug, *Murgantia histrionica* (Hemiptera, Heteroptera, Pentatomidae).

5.2 MATERIALS AND METHODS

5.2.1 Rearing of insects

Adults of *A. hilare* used to start the colony were provided by Kent Daane (University of California, Kearney Agricultural Center, Parlier, CA) and transferred to the our laboratory at the University of California at Berkeley (UCB) during the summer of 2006. *Murgantia histrionica*'s egg masses were shipped from Jocelyn Millar laboratory at the University of California at Riverside to UCB. We reared adults of both species in wood square cages inside an insectary room at 23±2ºC and photoperiod of 16:10 (L:D). Three sides of the cage were made of cloth-mesh material. Females used the cloth-mesh wall and strips of paper towel that we hung around the cage as oviposition surfaces. The colonies were cleaned and the food was replaced twice a week or when necessary. *Acrosternum hilare*'s diet consisted of green beans, broccoli, and raw peanuts, and *M. histrionica*'s diet consisted of broccoli and cauliflower. Egg masses from lab colonies were used to initiate the experiments. For all the experiments insects were placed in plastic containers lined with filter paper and fed only on green beans or broccoli, respectively, for *A. hilare* and *M. histrionica*. Adults were left in the plastic containers and allowed to lay eggs. Egg masses were collected every five days and placed in plastic containers to start the next generation.

5.2.2 Effect of egg mass structure on *A. hilare*'s mortality, nymphal development and reproduction parameters

We set up a preliminary experiment to test the effect that our surface sterilization treatment has on egg mass structure of *A. hilare*. This was done because some authors proposed that separation of the eggs during sterilization would increase mortality and affect insect's development (Lockwood and Story 1985). We tested three different treatments (five egg masses/treatment): surface sterilized, physically separated and intact egg masses. The surface sterilized treatment consisted of dipping the egg masses for 5 minutes in alcohol and 7 minutes in 10% bleach approximately two days before hatch. The next treatment consisted of physically separating the eggs two days before the insects hatched. We used the intact egg mass as a control treatment. We censused the insects every five days after hatch and calculated nymphal mortality, development time and the demographic parameters: net reproductive rate (R_0), mean generation time (T), intrinsic rate of increase (r), finite rate of increase (λ), doubling time (DT), and gross reproductive rate (GRR) as described in chapter 4 (Carey, 1993). In addition, we calculated adult emergence (day that the first adult appeared) and several oviposition parameters (pre-oviposition period, oviposition

period, number of egg masses) (Chapter 4). We evaluate symbiont retention by detecting the symbiont on egg masses in all treatments, two 5th instar nymphs, and an adult male and female using specific primers (see below).

5.2.3 *Acrosternum hilare* and *M. histrionica* experiments

We surface sterilized twenty egg masses of *A. hilare* as described above. We used twenty egg masses as a control. We censused the insects every five days on both treatments in order to calculate nymphal development time, survivorship and the demographic parameters: R_0, T, r, λ, DT, GRR, adult emergence (day that the first adult appeared), pre-oviposition period, oviposition period, and number of egg masses as described earlier (Carey, 1993, Chapter 4). Egg masses, two 5th instar nymphs, an adult female and male were used for detection of the symbiont in samples. We statistically analyzed the proportion of positive insects for the symbionts maintenance using a 2x3 contingency table (Crawley 2005). Chi-square tests were used to assess the significance of the differences between control and surface sterilized treatment within each of 3 stages: egg masses, nymphs and adults (Crawley 2005).

For *M. histrionica*, we surface sterilized fifteen egg masses as described above and used fifteen egg masses as a control in the first generation. For the second generation, we collected fourteen egg masses that originated from the control treatment and nine egg masses that originated from the surface sterilized treatment. We censused the insects every 5 days and evaluated nymphal development rate, survivorship and the biological parameters as describe above (Carey, 1993, Chapter 4). One egg mass, two 3rd instar nymphs, and an adult male and female were used for DNA extraction and detection of the symbiont (see below). Statistical analyses were conducted as above.

5.2.4 Diagnostic PCR

We extracted DNA from whole insects using a commercial kit (Qiagen, Valencia, CA) as previously described (Prado et al. 2006). Specific primers were designed based on the symbiont's partial 16SrRNA gene sequence (Prado and Almeida 2008). We designed the forward primer SP-Ah1 (5'-GAGGCTTAATACGCTTCG-3') and the reverse primer SP-Ah2 (5'-CAAGGAAACAACCTCCAG-3') for *A. hilare* and the forward primer SP-Mh1 (5'-CGCATAATGTTTCACAACC-3') and the reverse primer SP-Mh2 (5'-CACATCTCAAGGATACAAC-3') for *M. histrionica*. We used the same PCR cycles as previously described (Prado et al. 2006), except that the melting temperature for both of these primer sets was 58ºC.

5.2.5 Effect of egg mass surface sterilization on *A. hilare* and *M. histrionica* fitness:

We measured nymphal developmental time and calculated the median development time (MDT) as described by Peterson and Painting (1990). We executed all the statistical analyses using the software R v. 2.6.1 (R Development Core Team 2007). For the MDT statistical analyses, we used a linear mixed effects model with development stage as a random, repeated measure (Crawley 2005). We used a Cox proportional hazards model with censoring of the individuals that were removed for PCR to calculate the survival analysis. We compared Kaplan-Meier survival analysis for control and surface sterilized treatments in both *A. hilare* and *M. histrionica*. We calculated the biological parameters R_0, T, r, λ, DT, GRR, adult emergence, pre-oviposition period, oviposition period, and number of egg masses (Carey, 1993, Chapter 4). We calculated confidence intervals rather than ANOVA to compare *A. hilare*'s biological parameters between control and surface sterilized treatments because in the surface sterilized treatment we had only one repetition for the comparisons. For *M. histrionica* all the demographic and oviposition parameters were statistically analyzed using separate one-away ANOVA with 3 treatment levels (Control-Generation 1, Control-Generation 2, and Surface Sterilized-Generation 1). We used one-way ANOVA to compare the total number of eggs laid per female in 3 treatments: *A. hilare*-Control, *M. histrionica*-Control and *M. histrionica*-surface sterilized. We also used one-way ANOVA to compare the total number of eggs laid per female of *M. histrionica* in control and surface sterilized treatments during two generations. We used pairwise *t*-tests to compare means among *A. hilare*-Control, *M. histrionica*-Control and *M. histrionica*-surface sterilized.

5.3 RESULTS

5.3.1 Effect of egg mass structure on *A. hilare*'s mortality, nymphal development and reproduction parameters

We tested an egg mass surface sterilization method previously used for *N. viridula* (Prado et al. 2006) to sterilize *A. hilare* egg masses for two generations. Egg masses, two 5th instar nymphs and one adult male and one adult female were submitted to detection of the symbiont by PCR (see appendix A). Nymphal stage and cumulative mortality (see appendix B), mean development time (see appendix C), and demographic parameters (see appendix D and E) were calculated based on censused every day. The results indicated that the protocol needed to be optimized to be used efficiently for *A. hilare*, as sterilization method was not efficient. We modified the protocol by increasing the 5 minutes soak in 10% bleach for egg

masses to 7 minutes. The longer bleach wash caused the eggs in the masses to separate. Here we analyzed the effects of the physically individualized eggs and surface sterilization of eggs on *A. hilare*'s development, hypothesizing that egg mass structure does not impact stink bug fitness. We did not detect any significantly effect on survivorship of the physical separation of *A. hilare*'s eggs. Insects that originated from the surface sterilization treatment took longer to development from 1st to 5th nymphal instar (t = -2.181; d.f. = 41; p = 0.035) (Figure 5.6.1). In addition, symbiont retention, nymphal mortality and development, the demographic and reproductive parameters: R_0, T, r, λ, DT, GRR, adult emergence, pre-oviposition period, oviposition period, and number of egg masses did not differ among control and separated eggs (see appendix F, G, H, and I). These results support our hypothesis that, under our experimental conditions, egg mass structure has no impact on *A. hilare* biology (see additional information in appendices).

5.3.2 *Acrosternum hilare* and *M. histrionica* experiments

After a 7 min surface sterilization of eggs, in a preliminary test none of the 91 2nd instar insects tested at the surface sterilization treatment did not have the symbiont and 100% (n = 108) of control insects of *A. hilare* tested positive. After adjustment we detected *A. hilare*'s symbiont in 100, 87.9 and 100% respectively for egg mass, nymphs and adults tested (Figure 5.6.2 - A). In the surface sterilized treatment we detected *A. hilare*'s symbiont only in 30, 9.1 and 0% respectively for egg mass, nymphs and adults, which was significantly lower than the control for nymphs (χ^2 = 13.61, d.f. = 1, p < 0.001) and adults (χ^2 = 15.77, d.f. = 1, p < 0.0001).

Symbiont presence for *M. histrionica* is shown in Figure 5.6.2 - B. Overall, there was a significant effect of life stage (z = -2.813; p = 0.0049) and treatment (z = 5.096; p < 0.0001) on symbiont maintenance. We detected significantly higher infection rate of *M. histrionica*'s eggs in the control treatment than the surface sterilized egg masses only in the second generation (χ^2 =9.22; d.f. = 1; p = 0.0024). In nymphs, we detected significantly higher infection rate of the symbiont in the control than the surface sterilized treatment in both the first (χ^2 = 6.74; d.f. = 1; p = 0.00945) and second (χ^2 = 11.55; d.f. = 1; p = 0.0007) generation. In addition, adults of the control treatment had a significantly higher infection rate than at the surface sterilized treatment (χ^2 = 4.43, d.f. = 1, p = 0.035) during the first generation.

5.3.3 Effect of egg mass surface sterilization on *A. hilare* and *M. histrionica* fitness

Acrosternum hilare's nymphal development time was significantly longer in the surface sterilized egg mass treatment (108.84±12.65 days) than in the control treatment (54.08±0.63 days) (t = -4.02; d.f. = 158; p < 0.0001) (Figure 5.6.3 - A). *Murgantia histrionica*'s nymphal development time in the surface sterilized treatment was not significantly different of the control in the first or second generation (t = 0.45; d.f. = 202; p = 0.65) (Figure 5.6.3 - B). Statistical analyses of *A. hilare*'s and *M. histrionica*'s survivorship curves showed a significant effect of surface sterilization treatment (z = 6.291; p < 0.0001), and a significant interaction between the sterilization treatment and species (z = -5.440; p < 0.0001). The survival of *A. hilare* was negatively affected by the absence of its symbiont (χ^2 = 46; d.f. = 1; p < 0.0001) whereas the survival of *M. histrionica* was not affected by the absence of the symbiont (Figure 5.6.4). *Acrosternum hilare* reproduction was negatively affected by surface sterilization of the egg masses (Table 5.5.1). From the initial 20 egg masses from *A. hilare* used in the surface sterilized experiment only three females developed, and one female followed laid only one egg mass (Figure 5.6.5). In the control treatment, from the initial 20 egg masses used a total of 38 females of *A. hilare* laid 147 egg masses. For *M. histrionica* from 12 egg masses followed in the control treatment 16 females laid 69 egg masses at the first generation and at the second generation 5 females developed and laid 16 egg masses. In surface sterilized treatment from a total of 14 egg masses initially followed 14 females laid 53 egg masses for the first generation and all 6 females developed at the second generation were used for detection of the symbiont. *Murgantia histrionica*'s survivorship was slightly increased by absence of its symbiont (χ^2 = 5.8; d.f. = 1; p < 0.0163) (Figure 5.6.4). Comparisons between treatments and generations on *M. histrionica* survivorship demonstrated significant differences between generations (z = 2.31; p < 0.021) but not treatment (z = -1.42; p = 0.150). *Murgantia histrionica*'s mean generation time (T) was statistically longer in the surface sterilized treatment (t = 2.66; d.f. = 15; p = 0.0018) than in the control treatment (Table 5.5.1). In general, *M. histrionica*'s second generation on the control treatment showed a tendency of longer T and DT with smaller values of R_0, r, λ and GRR. As shown in the appendix J, *M. histrionica*'s pre-oviposition time was longer (F = 7.98; d.f. = 15; p = 0.0044) for the second generation of the control treatment than first generation and surface sterilized treatment. We also plotted the total number of eggs/female laid during *A. hilare* and *M. histrionica*'s adulthood development (Figure 5.6.5). Comparing three treatments, *A. hilare* – Control, *M. histrionica* - Control and *M. histrionica* - Surface sterilized, we showed that *A. hilare*'s capacity to lay eggs was higher at the control treatment when compared with *M. histrionica* control and surface sterilized treatment (F = 20.08; d.f. = 2; p < 0.0001).

5.4 DISCUSSION

We studied the effect of egg mass surface sterilization on the development and maintenance of *A. hilare* and *M. histrionica*'s gut-associated symbionts. Determining that the stink bugs *A. hilare* and *M. histrionica* were differently affected by surface sterilization of the egg masses was unexpected, and strongly suggests that different levels of mutualism may exist in these associations for the two insects. In *A. hilare*, the absence of the gut symbiont negatively impacted nymphal development, survivorship and reproduction. In addition, we observed higher mortality of the surface sterilized insects of *A. hilare* mainly during the first and second instars, and few insects completed development to become adults. This explains the low values of the biological parameters R_0, T, r, λ, DT, GRR. In *A. hilare*, the high mortality observed in our studies suggests that the symbiont may play an important physiological role, providing *A. hilare* with essential nutrients. For *M. histrionica*, surface sterilization of eggs and consequent deprivation of its gut-associated symbiont did not show any clear effect on nymphal developmental time (unlike *A. hilare*), and surface sterilization of eggs masses caused a slight increase in host survival in the first generation. Additionally, we did not detect any significant difference in *M. histrionica*'s demographic parameters, except that mean generation time was longer for the surface sterilized treatment in the first generation. The natural infection showed in control results of *M. histrionica*'s symbiont in the insects was lower than for *A. hilare*'s symbiont (~100%) in the first generation (~80%) and in the second generation (~60%), and the surface sterilization method used for *M. histrionica* was less effective. The lower proportion of *M. histrionica* insects carrying the symbiont in the second generation may have affected the insect's development and reproduction measured here. We used the same surface sterilization technique for *M. histrionica* as for *A. hilare* without further optimization, and this technique probably was not sufficient to clear all the symbionts within study insects. The detection of positive *M. histrionica* in the surface sterilized treatment influenced the interpretation of the results. The fact that there were no starter cohorts produced, which resulted in no data being collected for *M. histrionica* demographic parameters for the second generation could suggest that *M. histrionica* is affected by the symbiont absence. However, the apparent success of the first generation with more symbiont-positive insects and the subsequent failure of the second generation for surface sterilized insects suggest that *M. histrionica* may be benefited by hosting the symbiont, but the sterilization method used was not adequately efficient to have a significant effect. It is clear that further work testing different exposure time and concentrations of bleach or different treatments to surface sterilize *M. histrionica*'s egg masses is necessary. We previously showed that *N. viridula*'s biology is not affected by deprivation of its gut symbiont under similar lab conditions (Prado et al. 2006, Prado et al. unpublished); however Abe et al (1995) showed that sterilization of egg masses negatively affected

P. stali. Thus, these results corroborate previous reports in the literature and suggest that each insect species in the family Pentatomidae may have a unique relationship with its gut-associated symbiont.

The surface sterilization method used previously for *N. viridula* had to be adjusted to efficiently sterilize *A. hilare* egg masses. Although some surface sterilized egg masses (n = 6) and nymphs (n = 3) were positive for *A. hilare*'s symbiont, none of the 22 adults analyzed were positive. Furthermore, the results of our preliminary test showed that the separation of the eggs that occurred during the surface sterilization of the egg masses has no effect on mortality or on the insect's development as some authors proposed earlier (Lockwood and Story 1985). These results also show that the methods used should be carefully optimized to prevent spurious outcomes or interpretation of experiments.

Several papers discussing the life cycles of *M. histrionica* and *A. hilare* and reproduction have been published (Canerday 1965, Ludwig and Kok 2001, Simmons and Yeargan 1988, Streams and Pimentel 1963, Zahn et al. 2008). There are some disparities in *A. hilare* and *M. histrionica*'s development and demographic parameters reported in the articles cited and the results presented in this study. This is probably the result of using different experimental methods, including the intervals that the insects where censused at, and/or different diets used. We fed *A. hilare* with green beans and peanuts which are rich in fat and proteins; however *M. histrionica* requires longer ingestion time when fed on broccoli and this could affect its development (Todd 1989, Zahn et al. 2008). Additionally, the slower development observed in our study may be due to the reduced viability and fecundity of the insects reared for successive generations without addition of new insects to the colonies (Harris and Todd 1980, 1981). Importantly, future research utilizing different and standardized diets to rear insects from surface sterilized eggs can provide further information on the nutritional role of the symbionts in these host insects. Considering the polyphagous nature of the pentatomids, the diet tested should mimic the complex nutritional environment typically exploited by the insects.

There seems to be variability in the dependence of stink bug hosts on their respective gut symbionts. *Acrosternum hilare* and *P. stali* require their symbionts to accrue any benefit from the association; *M. histrionica*, although not irrefutably proven, seems to have an intermediate or facultative relationship, while *N. viridula* seems to not require its symbiont to live (Prado et al. 2006, Prado et al. unpublished). The different response to the absence of symbionts in each respective host development may be explained by the polyphyletic origin of the symbionts associated with Pentatomidae. Different species of pentatomid insects have different levels of association with their symbionts. This is probably due to the unique symbiont transmission mode which allows the symbiont to live for a short period of time

outside its host. The fact that the symbionts are not obligatory for the host's survival and development doesn't necessary mean that they are not important. Recently, Hosokawa et al (2007) showed that the symbiont of *M. punctatissima* is involved in the host's ability to feed on a certain crop plants and may be influencing the evolution of new pest insects. It remains unknown whether the success of pentatomid insects as pests worldwide is related to the presence of their caeca-associated symbionts. These results contribute to the sparse data available on symbionts of pentatomid insects as well as other heteropteran insects and remain interesting hypotheses for further investigation, considering other species of pentatomid insects and/or different diets and different levels of pest status and polyphagy.

5.5 TABLES

Table 5.5.1. Effect of egg mass surface sterilization on demographic parameters of *Acrosternum hilare* and *Murgantia histrionica.*

Species	Treatment/ Generation	n/n[1]	R_0 (♀/♀)[2]	T (days)[3]	r (♀/♀/day)[4]	λ (♀/♀/day)[5]	DT (days)[6]	GRR[7]
A. hilare	Control/ G1	11/38	21.59±7.71[8]	135.99±19.56	0.02±0.004	1.02±0.004	34.48±7.87	81±20.79
	SS[9]/G1	1/1	0.40	197	-0.005	0.995	-[10]	4
M. histrionica	Control/ G1	8/16	12.17±2.94[11]a[12]	128.49 ±14.52a	0.02±0.003a	1.02±0.003a	54.33±15.32a	31±6.04a
	Control/ G2	4/5	6.19±1.99a	147.71±7.21a	0.01±0.003a	1.01±0.003a	114.70±63.11a	17±4.46a
	SS/G1	6/14	9.39±1.60a	185.16±19.09b	0.01±0.001a	1.01±0.001a	60.31±3.65a	25±3.74a
	SS/G2	0/0	-	-	-	-	-	-

[1] Number of cohorts used for the calculation of the parameters/total number of females by the time of the first oviposition in the replicates.

[2] $R_0 = \sum l_x m_x$ - net reproductive rate.

[3] $T = (\sum x\, l_x m_x)/R_0$ - mean generation time.

[4] $r = ln(R_0)/T$ - instantaneous rate of increase.

[5] $\lambda = exp\ (r)$ - finite rate of increase.

[6] $DT = ln2/r$ - population doubling time.

[7] $GRR = \sum m_x$ - gross reproductive rate.

[8] Values of *A. hilare* = median ± confidence interval (95% CI, n = 11).

[9] SS = surface sterilized.

[10] – no data were collected.

[11] Values of *M. histrionica* = Median ± standard error (SE).

[12] Means followed by the same letters in the same column are not significantly different ($P<0.05$).

5.6 FIGURES

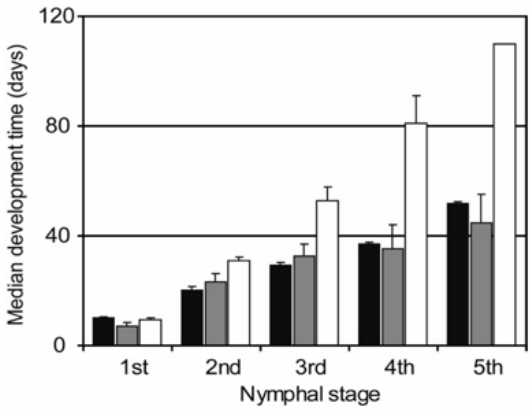


Figure 5.6.1. Median nymphal development time (+SE) of *Acrosternum hilare* comparing the role of egg mass integrity on insect development. Entire egg mass (black column), physically broken egg mass (grey column), and surface sterilized egg mass (white column).

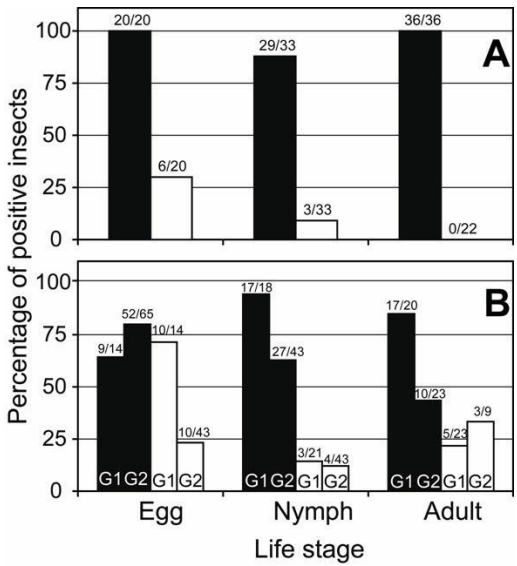


Figure 5.6.2. Percentage of symbiont positive individuals for *Acrosternum hilare* (A) and *Murgantia histrionica* (B). Black columns represent the control treatment and white columns represent the surface sterilized treatment. G1 = fist generation and G2 = second generation. Numbers above columns represent the proportion of positive insects by PCR/total tested.

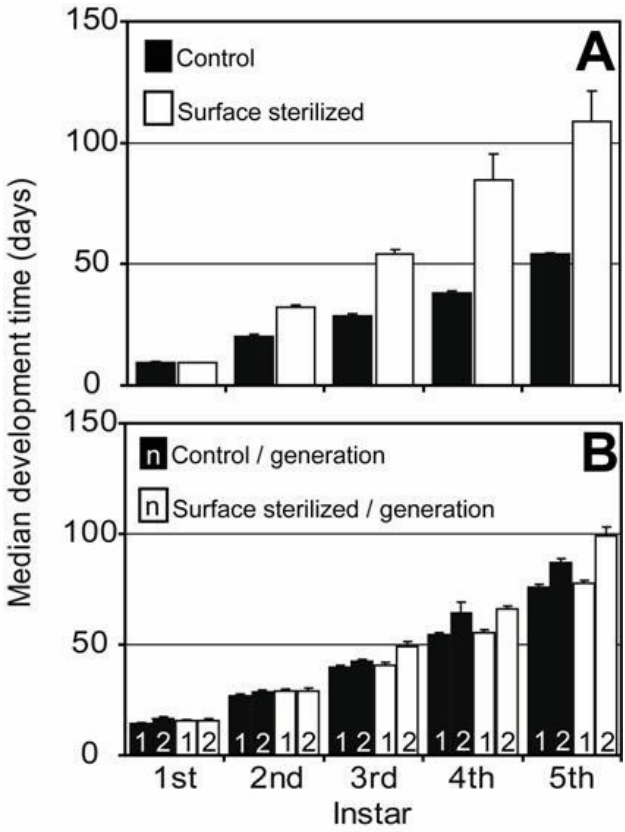


Figure 5.6.3. Mean nymphal development time (+SE) in the 5 nymphal stages of *Acrosternum hilare* (A) and *Murgantia histrionica* (B). Black columns represent the control treatment and white columns represent the surface sterilized treatment. Numbers in the columns indicates generation.

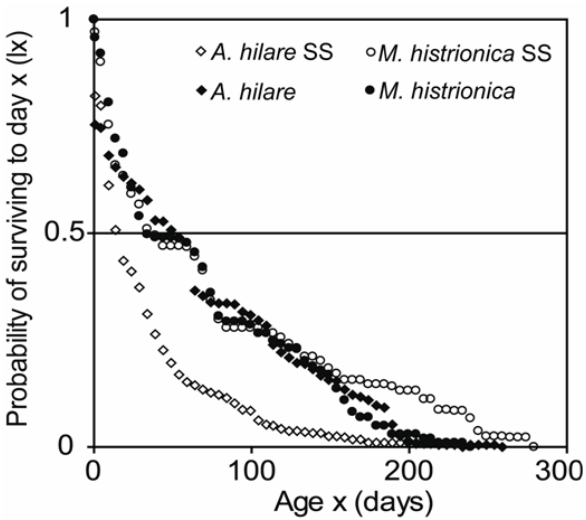


Figure 5.6.4. Survivorship curves for *Acrosternum hilare* and *Murgantia histrionica*. Black symbols represent control treatment and white symbols represent surface sterilized (SS).

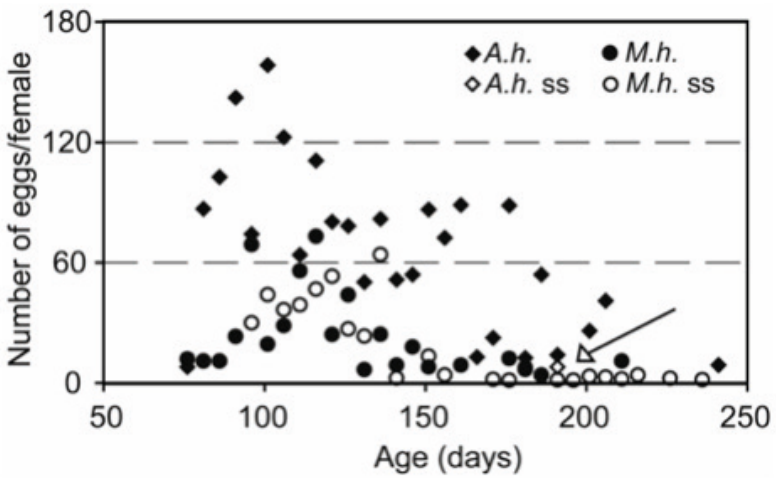


Figure 5.6.5. Total number of eggs laid per female of *Acrosternum hilare* and *Murgantia histrionica* during the first generation in the control and surface sterilized (ss) treatments. The arrow indicates the only egg mass laid in the surface sterilized treatment of *A. hilare*.

LITERATURE CITED

Abe, Y., Mishiro K., and M. Takanashi. 1995. Symbiont of brown-winged green bug *Plautia stali* Scott. Jpn. J. Appl. Entomol. Zool. 39: 109-115.

Baumann, P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-suking insects. Annu. Rev. Microbiol. 59: 55-89.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers, New York.

Canerday, T.D. 1965. On the biology of the harlequin bug, *Murgantia histrionica* (Hemiptera: Pentatomidae). Ann. Entomol. Soc. Amer. 58: 931-932.

Carey, J.R. 1993. Applied demography for biologists with special emphasis on insects. Oxford University Press, Oxford, England.

Crawley, M.J. 2005. Statistics: An introduction using R. John Wiley & Sons, England.

Douglas, A.E. 1996. Reproductive failure and the amino acid pools in pea aphids (*Acyrthosiphon pisum*) lacking symbiotic bacteria. J. Insect Physiol. 42: 247-255.

Durvasula, R.V., Kroger, A., Goodwin, M., Panackal, A., Kruglov, O., Taneja, J., Gumbs, A., Richards, F.F., Beard, C.B., and C. Cordon-Rosales. 1999. Strategy for introduction of foreign genes into field populations of chagas disease vectors. Ann. Entomol. Soc. Amer. 92: 937-943.

Fukatsu, T., and T. Hosokawa. 2002. Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. Appl. Environ. Microbiol. 68: 389-396.

Harris, V.E., and J.W. Todd. 1980. Comparative fecundity, egg fertility and hatch among wild-type and three laboratory-reared generations of the southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae). J. Georgia Entomol. Soc. 15: 243-253.

Harris, V.E., and J.W. Todd. 1981. Rearing the southern green stink bug, *Nezara viridula*, with relevant aspects of its biology. J. Georgia Entomol. Soc. 16: 203-210.

Hirose E., Panizzi, A.R., De Souza, J.T., Cattelan A.J., and J.R. Aldrich. 2006. Bacteria in the gut of southern green stink bug (Heteroptera: Pentatomidae). Ann. Entomol. Soc. Am. 99:91-95.

Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., and T. Fukatsu. 2006. Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. PLoS Biology 4: 1841-1851.

Hosokawa, T., Kikuchi, Y., Shimada, M., and T. Fukatsu. 2007. Obligate symbiont involved in pest status of host insect. Proc. R. Soc. B. 274: 1979-1984.

Kikuchi, Y., Hosokawa, T., and T. Fukatsu. 2007. Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. Appl. Environ. Microbiol. 73: 4308-4316.

Kikuchi, Y., Meng, X.Y., and T. Fukatsu. 2005. Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Hemiptera: Alydidae). Appl. Environ. Microbiol. 71: 4035-4043.

Lawyer, P.G., and P.V. Perkins. 2000. Leishmaniasis and trypanosomiasis. Medical Entomology. B. F. Eldridge and Edman, J. D. Netherlands, Kluwer Academic Publishers: 231-298.

Lockwood, J.A, and R.N. Story. 1985. Photic, thermic, and sibling influences on the hatching rhythm of the southern green stink bug, *Nezara viridula* (L.). Environ. Entomol. 14: 562-567.

Ludwig, S.W., and L.T. Kok. 2001. Harlequin bug, *Murgantia histrionica* (Hahn) (Heteroptera: Pentatomidae) development on three crucifers and feeding damage on broccoli. Crop Protec. 20: 247-251.

Moran N.A., and A. Mira. 2001. The process of genome shrinkage in the obligate symbiont *Buchnera aphidicola*. Genome Biol. 2:research0054.1-0054.12

Moran, N.A., Munson, M.A., Baumann, P., and H. Ishikawa. 1993. Molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. Proc. R. Soc. Lond. B. 253: 167-171.

Munson, M.A., Baumann, P., Clark, M.A., Baumann, L., Moran, N.A., Voegtlin, D.J., and B.C. Campbell. 1991. Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. J. Bact. 173: 6321-6324.

Panizzi, A.R., McPherson, J.E., James, D.G., Javahery, M., and R.M. McPherson. 2000. Economic importance of stink bug (Pentatomidae). In Schaefer C.W. and A.R. Panizzi [eds.], Heteroptera of economic importance, CRC, Boca Raton, Florida.

Peterson, W.T., and S.J. Painting. 1990. Developmental rates of the copepods *Calanus australis* and *Calanoides carinatus* in the laboratory, with discussion of methods used for calculation of development time. J. Plankton Res. 12: 283-293.

Prado, S.S., and R.P.P. Almeida. 2008. Phylogenetic placement of pentatomid stink bug gut symbionts. Curr. Microbiol. Accepted.

Prado, S.S., Rubinoff, D., and R.P.P. Almeida. 2006. Vertical transmission of a pentatomid caeca-associated symbiont. Ann. Entomol. Soc. Am. 99: 577-585.

R Development Core Team 2006 R: a language and environment for statistical computing. Vienna, Austria: R Foundation for statistical computing. See http://www.R-project.org

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. and H. Ishikawa. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. Nature 407: 81-86.

Simmons, A.M., and K.V. Yeargan. 1988. Development and survivorship of the green stink bug, Acrosternum hilare (Hemiptera: Pentatomidae) on soybean. Environ. Entomol. 17: 527-532.

Streams, F.A., and D. Pimentel. 1963. Biology of the harlequin bug, *Murgantia histrionica*. J. Econ. Entomol. 56: 108-109.

Tamas, I., Klasson, L.M., Canback, B., Naslund, A.K., Eriksson, A.-S., Wernegreen, J.J., Sandstrom, J.P., Moran, N.A., and S.G.E Anderson. 2002. 50 million years of genomic stasis in endosymbiotic bacteria. Science 296: 2376-2379.

Todd, J.W. 1989. Ecology and behavior of *Nezara viridula*. Ann. Rev. Entomol. 34: 273-292.

Zahn, D.K., Girling, R.D., McElfresh, J.S., Carde, R.T., and J.G. Millar. 2008. Biology and reproductive behavior of *Murgantia histrionica* (Heteroptera: Pentatomidae). Ann. Entomol. Soc. Am. 101: 215-228.

CHAPTER 6

EFFECT OF TEMPERATURE ON THE MAINTENANCE OF PENTATOMID GUT-ASSOCIATED SYMBIONTS

Simone de Souza Prado

ABSTRACT: *Acrosternum hilare* (Say) and *Murgantia histrionica* (Hahn) (Hemiptera, Pentatomidae) have vertically transmitted bacterial symbionts in their midguts. We determined the impact of temperature on the biology of both insects and their association with their respective gut-associated symbionts. Data on nymphal mortality, developmental time, and symbiont presence were taken for both species at 25˚C and 30˚C. *Acrosternum hilare* showed higher mortality in the 1st and 5th instars at 30˚C (26% and 37%, respectively) when compared with 1st and 5th at 25˚C (9% and 3%, respectively), but its development time was not altered. *Acrosternum hilare* reared at 25˚C remained infected with the gut symbiont until the adult stage of the second generation in our tests. Those reared at 30˚C were PCR positive 25% of 5th instars during the first generation, at the second generation from the 2nd instar until adulthood none of the insects were positive by PCR. *Murgantia histrionica* also had similar developmental time at 25 and 30˚C, however we only observed higher mortality for its second generation at 30˚C when compared with 25˚C. *Murgantia histrionica* had lower rates positive insects for the symbiont at 25˚C than *A. hilare*. A total of 68% of egg masses and 73.3% of 5th instars were positive for *M. histrionica*'s gut symbiont at 25˚C. At 30˚C 27% of the egg masses were positive, but all 5th instars and adults of *M. histrionica* were negative for the symbiont at 30˚C. Our data show that increased temperature affect the fitness of two pentatomid species and eliminate gut-associated bacteria in these insect.

6.1 INTRODUCTION

Microbial symbiosis is of importance to a diversity of arthropods. Although it was recognized over 50 years ago that insects of the suborder Heteroptera (order Hemiptera) have intimate symbiotic associations with bacteria in their gut, for most families the significance of such relationships and their role in the host's ecology and evolution remain unknown (Buchner 1965, Baumann 2005).

There are numerous strategies that insects use for the symbiont vertical transmission of symbionts in the Heteroptera, but only a few of these systems have been studied in detail (Buchner 1965, Durvasula et al. 1999, Fukatsu and Hosokawa 2002, Hosokawa et al. 2006, Kikuchi et al. 2005, Prado et al. 2006). For example, the assassin bug *Rhodnius prolixus* (Stal) (Reduviidae) harbors the bacterial symbiont *Rhodococcus rhodnii* in its gut lumen (Buchner 1965, Durvasula et al. 1999). In this family, the aposymbiotic (without symbiont) first instar nymphs hatch and probe on fecal pellets deposited by adults to acquire the symbiont orally (Durvasula et al. 1999). Durvasula et al. (2003) explored the symbiotic relationship between *R. prolixus* and *R. rhodnii* by transforming the symbiont to negatively interfere on the survivorship of the Chaga's disease agent, *Trypanosoma cruzi*. Fukatsu and Hosokawa (2002), studying bugs in another family, showed that the plataspid insect *Megacopta punctatissima* (Montandon) harbors the bacterial symbiont '*Candidatus* Ishikawaella capsulata' in its gastric caeca. After hatching, aposymbiotic first instar nymphs immediately probe on small brownish capsules attached to the eggs masses laid by the females to acquire the symbiont (Fukatsu and Hosokawa 2002, Hosokawa et al. 2006, Hosokawa et al. 2007). In addition, Hosokawa et al (2006) used phylogenetic reconstruction to show that both insect and symbiont have undergone cospeciation, and when deprived of its symbiont, *M. punctatissima*'s growth and survival are deleteriously influenced (Fukatsu and Hosokawa 2002, Hosokawa et al. 2006, Hosokawa et al. 2007). On the other hand, the insects *Riptortus clavatus* (Thunberg) and *Leptocorisa chinensis* (Dallas) (Alydidae) harbor symbionts on the genus *Burkholderia*. Those insects acquire these horizontally transmitted symbionts from the soil each generation (Kikuchi et al. 2005, 2007).

In the family Pentatomidae, insects have been shown to harbor dominant *Erwinia*-like bacterial symbionts associated with the gastric caeca region of their midguts (Prado and Almeida 2008). Smearing of the symbiont on egg surfaces by females while ovipositing and subsequent acquisition of the symbiont by aposymbiotic first instar nymphs appears to be the mechanism used for vertical transmission of pentatomid caeca-associated symbionts in *N. viridula*, *A. hilare*, *M. histrionica* and *Thyanta pallidovirens* (Stal) (Prado et al. 2006, Prado et al. unpublished, S. Prado personal observation). *Nezara viridula* is not clearly affected by the removal of their symbionts when eggs are submitted to surface sterilization at 25ºC (Hirose et al. 2006, Prado et al. 2006), but at 30ºC *N. viridula*'s symbiont maintenance is affected and insect development is fastened (Prado et al. unpublished). In addition, *Plautia stali* Scott, when deprived of its gut-associated symbiont, has slower development time than individuals with the symbiont (Abe et al. 1995). For *A. hilare*, the elimination of the symbiont by surface sterilization of the egg masses negatively impacted development and reproduction (Prado et al. unpublished). Conversely, absence of *M. histrionica*'s gut symbiont seems to have no effect on the development of the insect host. The role of these vertically transmitted pentatomid gut symbionts therefore appears to vary for different bug host species.

Various abiotic factors are known to influence development of insects and their associations with microorganisms. High temperature is one of those factors. For example, aphid reproduction is negatively impacted at high temperature (37ºC) after elimination of the primary endosymbiont, *Buchnera aphidicola* (Ohtaka and Ishikawa 1991, Chen et al. 2000). On the other hand, facultative symbionts may confer aphids fitness benefits such as heat stress tolerance (Montllor et al. 2002, Russell and Moran 2006), and compensation for loss of *Buchnera* (Koga et al. 2003). Despite the importance of temperature on the maintenance of insect symbionts and their relationship with their hosts, a limited number of insect taxa have been studied in this regard. We investigated effects of the temperature on the maintenance of *A. hilare* and *M. histrionica*'s gut symbiont and in the in the biology of the pentatomid host.

6.2 MATERIALS AND METHODS

6.2.1 Colonies

Adults of *A. hilare* and egg masses of *M. histrionica* were kindly provided by Kent Daane and Jocelyn Millar, respectively to initiate a lab colony at UC Berkeley, CA. We used a diet that consisted of green beans, broccolis and raw peanuts to rear *A. hilare*. For *M. histrionica*, we used broccoli and cauliflower to feed the insects. We supplied the colonies with fresh food about twice a week. Both species were reared in a walk-in insectary room at 24±2ºC and photoperiod of 16:10 (L:D). We collected the egg masses from both colonies to initiate the experiments. *Acrosternum hilare* was fed green beans for nymphs and of green beans and peanuts for adults during the experiments and *M. histrionica* was fed broccoli for nymphs and adults.

6.2.2 Effect of temperature on *A. hilare* and *M. histrionica* biology

Experiment I. We set up an initial experiment to test if high temperature affected symbiont maintenance in *A. hilare*. For this experiment we collected thirty egg masses from the *A. hilare* colony and placed each of them in plastic containers covered with lids glued with mesh cloth for air circulation. Fifteen of them were placed in a controlled temperature chamber at 25ºC while the other fifteen eggs masses were placed in a controlled temperature chamber at 30ºC. Both environments had a consistent photoperiod of 16:8 L/D. We added fresh green beans every other day. The insects were censused every five days taking in consideration its median development time, mortality and fecundity.

Experiment II. We also separated thirty egg masses of *A. hilare* from the colony and fifteen egg masses were placed each of both temperatures 25 and 30ºC, respectively. In this experiment we provided cohorts with new food every other

day, but we censused the insects every seven days taking in consideration the same parameters mentioned above. We transferred the adults to a separate cage and allowed them to breed in the same temperature and environment as their parents. To compose the second generation we collected thirty egg masses originated from the adults, and fifteen egg masses were placed in each of the two controlled temperature chamber as their parents and receive similar treatment with regard to the feeding and counting methods than the first generation.

Experiment III. A total of twenty egg masses of *M. histrionica* were placed in controlled chambers at 25 and 30ºC in the first generation. Each egg mass was confined in plastic containers and allowed to develop. When mature, the insects were allowed to mate and their egg masses were placed in new plastic containers in the same environment as their parents. During the second generation, about forty five egg masses were held at 25ºC and twenty egg masses were held at 30ºC. We replaced the food every other day and we censused all replications every three days during two generations.

6.2.3 Maintenance of *A. hilare* and *M. histrionica* symbionts

For the first experiment we collected an egg mass and two 5th instars nymphs of *A. hilare* from each rearing container to detect the symbiont by PCR as described earlier (Prado et al. 2006, chapter 5). For the second experiment two nymphs of 2nd, 3rd, 4th and 5th stages and one adult male and one female of *A. hilare* were sampled from each replicate for detection of the symbiont. In a third experiment we sampled one nymph of the 2nd, 3rd, 4th and 5th stages and one male and one female of *M. histrionica*. We statistically analyzed the second and third experiments using a 2x4 contingency table for each of the three stages separated: egg masses, nymphs and adults. The proportion of positive insects analyzed as the frequency of insects was used to assess the symbiont presence at 25 and 30ºC during 2 generations. We used chi-square goodness of fit tests to assess the significance of the differences between 25 and 30ºC within the stages: egg masses, nymphs and adults with adjusted $P < 0.018$ for significance in the comparisons. Follow-up chi-square tests between temperature and generations in each stage were done assuming adjusted $P < 0.00417$ for significance in the comparisons (Crawley 2005).

6.2.4 Demographic parameters and statistical analysis

In all experiments we calculated median development time (MDT) as previously described (Peterson and Painting 1990). The biological parameters: net reproductive rate (R_0), mean generation time (T), intrinsic rate of increase (r), finite rate of increase (λ), doubling time (DT), and gross reproductive rate (GRR) were calculate as described

by Carey (1993). We determined adult emergence (day first adult appeared) and the oviposition parameters (pre-oviposition period, oviposition period, number of egg masses and total number of eggs per egg masses) as described by Carey (1993).

All statistical analyses were done using the software R v. 2.6.1 (R Development Core Team 2007). We statistically analyzed mortality using a two-way ANOVA with temperature and generations as a fixed factor and stage or cumulative mortality as the response variable. Statistical analyses of median development time were performed using a linear mixed effects model with development stage as a random, repeated measure (Crawley 2005). The statistical analyses of the biological parameters for *M. histrionica* were calculated using a separate one-away ANOVA with 3 treatment levels (25ºC-Generation 1, 25ºC-Generation 2, and 30ºC-Generation 1). Pairwise *t*-tests were used to compare means among the treatments, with adjusted *P*-values for multiple comparisons. We also used one-way ANOVA to compare the total number of eggs laid throughout the first generation lifetime of *A. hilare* and *M. histrionica*.

6.3 RESULTS

6.3.1 Maintenance of *A. hilare* and *M. histrionica* symbionts

The initial experiment (I) showed that a 100% of the egg masses of *A. hilare* at 25ºC (15/15) and at 30ºC (14/14) were positive for the presence of the symbiont. For 5th instar a 100% (26/26) of the nymphs were positive at 25ºC; however 0% (0/22) were positive for the symbiont at 30ºC. To determine when the symbionts were lost, we followed the maintenance of *A. hilare*'s symbiont in the second experiment (II). We detected *A. hilare*'s symbiont in 87.8% to 100% of the insects tested at 25ºC in both generations (Figure 6.6.1). At 30ºC *A. hilare*'s symbiont was detected in 25.0 to 41.7% of the insects tested from second instar to adults during the first generation; however, in the second generation none of the insects tested positive for the symbiont. Statistical analysis showed a significant difference in the percentage of nymphs ($\chi^2 = 76.7$; d.f. = 3; $p < 0.0001$) positive for the symbiont during the first ($\chi^2 = 23.7$; d.f. = 1; $p < 0.0001$) and second generation ($\chi^2 = 49.9$; d.f. = 1; $p < 0.0001$) between 25ºC and 30ºC and at 30ºC of both generations ($\chi^2 = 14.9$; d.f. = 1; $p < 0.0001$), but not for eggs ($\chi^2 = 1.6$; d.f. = 3; $p = 0.665$) or adults ($\chi^2 = 8.8$; d.f. = 3; $p = 0.033$).

Experiment III examined the maintenance of *M. histrionica*'s gut symbiont. Results showed that the percentage of gut-symbiont positive insects for *M. histrionica* ranged from 62.5 to 81.2% during the first generation and from 31.2 to 46.9% during the second generation at 25ºC (Figure 6.6.1). At 30ºC, the *M. histrionica* symbiont was detected in 69.7, 56.5, and 36.8% respectively for 2nd, 3rd, and 4th instar nymphs and in none of the 5th instar and adults of this first generation and in none of the insects

evaluated during the following generation. Chi-square tests showed a significant difference between eggs ($\chi^2 = 23.9$; d.f. = 3; $p < 0.001$), nymphs ($\chi^2 = 39.8$; d.f. = 3; $p < 0.001$) and adults ($\chi^2 = 16.9$; d.f. = 3; $p < 0.001$) reared at 25 and 30ºC. Follow-up tests showed statistical differences on the presence of symbionts for eggs at 30ºC between first and second generations ($\chi^2 = 18.2$; d.f. = 1; $p < 0.0001$) and during second generation between 25 and 30ºC ($\chi^2 = 17.9$; d.f. = 1; $p < 0.0001$), within nymphs and adults. We observed statistical similar pattern in differences at 30ºC between first and second generations of *M. histrionica* and during second generation between 25 and 30ºC for nymphs ($\chi^2 = 22.3$; d.f. = 1; $p < 0.0001$) ($\chi^2 = 23.7$; d.f. = 1; $p < 0.0001$) and adults ($\chi^2 = \text{inf}$; d.f. = 1; $p < 0.0001$) ($\chi^2 = 13.4$; d.f. = 1; $p < 0.0003$) respectively for the presence of symbionts.

6.3.2 Demographic parameters and statistical analysis

Life stage and cumulative mortality for *A. hilare* and *M. histrionica* are shown in Figure 6.6.2. *Acrosternum hilare* mortality showed significant affects of temperature treatments ($t = -4.43$; d.f. = 305; $p < 0.0001$), generations ($t = -3.46$; d.f. = 305; $p = 0.0006$) and in the interaction between treatments and generations ($t = -3.23$; d.f. = 305; $p = 0.0014$), being higher at 30ºC. The statistical analyses did not show differences among stages, but there was a low viability of eggs and higher mortality at 1st and 5th nymphal stages during the first generation and at the first stages of the second generation. In addition, *A. hilare* cumulative mortality was significantly higher at 30ºC than at 25ºC ($F = 10.66$; d.f. = 1; $p = 0.0019$). For *M. histrionica* stage mortality showed significant interaction among treatments and generation ($t = -2.31$; d.f. = 547; $p = 0.0212$). *Murgantia histrionica* cumulative mortality showed significant affects of generation ($F = 8.54$; d.f. = 1; $p = 0.0045$) and treatments interaction ($F = 4.60$; d.f. = 1; $p = 0.035$).

Medium development time was not significantly different between *A. hilare* in experiments I and II (t=0.642; d.f. = 343; $p = 0.521$). We did not detect any effect of temperature ($t = 0.60$; d.f. = 337; $p = 0.55$) or species ($t = -0.13$; d.f. = 337; $p = 0.90$), or among the interaction between both species during two generations ($t = -0.59$; d.f. = 337; $p = 0.56$) (Table 6.5.1).

The biological parameters calculated for *A. hilare* and *M. histrionica* are presented on Table 6.5.2. For *A. hilare*, it is clear that high temperature affected the insect's development and reproduction. At 30ºC the low R_0 value resulted in a negative value of *r*, meaning that population was decreasing. In addition, we could not estimate the time for the population to double. *GRR* was also affected, resulting in less than half of the same *GRR* value obtained at 25ºC. Pre-oviposition period and number of eggs laid in each egg mass at 30ºC was lower than at 25ºC (Table 6.5.3). For *M.*

histrionica only *GRR* was significantly lower at 25ºC during the second generation. Age for the first adult appearance was faster at 30ºC in the first generation (Table 6.5.3).

Statistical analyses showed a significant higher number of eggs laid by all females of *A. hilare* at 25ºC ($F = 9.85$; d.f. = 2; $p < 0.0001$) than for females at 30ºC and than all females of *M. histrionica* at 25 and 30ºC (Figure 6.6.3).

6.4 DISCUSSION

The effects of temperature on the development of *A. hilare* and *M. histrionica* and maintenance of their gut-associated symbionts were studied. In general, our results demonstrated that temperature affects maintenance of the gut-associated bacteria found in the caeca of *A. hilare* and *M. histrionica*. The symbionts were not as prevalent in the insect gut at the 30ºC as compared to the 25ºC for both species. We showed that egg masses of *A. hilare* were 100% positive for the presence of the symbiont, but the 5th instars nymphs were 100% negative for the symbiont when the insects were reared at 30ºC. To determine when insects lost the symbionts we tested individual insects in each stage (except 1st instar). For *A. hilare*, during the first generation at 30ºC the proportion of insects with the symbionts were lower that the ones at 25ºC ranging from 42 to 25%, but from the 2nd instar through adults all insects sampled at the second generation were negative for the symbiont. These divergent results observed in the symbiont presence for 5th instars of *A. hilare* at 30ºC between first and second experiment can be explained by the later time that the egg masses were placed at 30ºC. Heating the eggs later may have not allowed enough exposure time for the endosymbionts on the egg surface be affected by the high temperature before the insects hatched. For *M. histrionica*, first generation insects reared at 30ºC had a symbiont infection rate approximately 2/3 that of those reared at 25ºC. However, in the 5th instar of the first generation through the second generation all insects sampled were negative for the symbiont. Clearly, high temperatures affect the maintenance of symbionts in pentatomid insects (Prado et al. unpublished); however further research quantifying the population of symbionts inside the insects is necessary. It will be important to determine with more sensitive detection methods if *M. histrionica*'s symbionts have a lower infection rate or if the symbionts are present in low populations that could not be detected by the standard PCR protocol used here.

At higher temperatures, in addition to loss of the symbiont, mortality of *A. hilare* increased, biological parameters R_0, *r, DT,* and *GRR* were negatively impacted, but its nymphal development time remained constant. The increased mortality observed for *A. hilare* mainly during 1st and 5th instar of the first generation and the 1st and

2nd instar of the second could be due to the absence of the symbiont and/or higher sensitivity of *A. hilare* to higher temperatures when its major symbiont is absent. At 30ºC for the first generation, results showed lower percentage of insects hatching and higher cumulative mortality independent of generations. At 30ºC, values of R_0 was 10 times lower, *GRR* was 2 times lower, *DT* could not be calculated, and *r* was negative which indicates that the population at 30ºC is been negatively affected and decreasing. Females of *A. hilare* had lower pre-oviposition period and laid fewer eggs at 30ºC when compared with 25ºC. In previous work, we showed that surface sterilization of the egg masses clear off the symbiont of *A. hilare*'s gut. Absence of symbionts resulted in increased nymphal mortality, and negatively impacts the insect's development (Prado et al. chapter 5). Results of this work suggest that *A. hilare* are negatively impacted when not harboring the symbiont and that the negative effect of high temperatures on symbiont survival observed here is not uncommon (Buchner 1965, Montllor et al. 2002).

Results for *M. histrionica* showed that a lower percentage of insects hatched at 30ºC in both generations. Mortality of *M. histrionica* at 30ºC was higher in the second generation, mainly in the 1st, 2nd and 4th instars, and probably this is related to the absence of the symbiont. The biological parameters were not statistically different, but at 30ºC tended to be lower than at 25ºC. *GRR* was statistically lower in the second generation held at 25ºC, and 111 adults emerged from 36 cohorts followed. The first adult emerged earlier at 30ºC during the first generation probably because of they accumulated degree days faster. Only 4 adults emerged in two out of 22 cohorts held at 30ºC during the second generation, and they were used for detection of the symbiont and could therefore not be used to quantify any of the biological parameters. Further work is need to clarify if the life table parameters at 30ºC during the second generation could not be calculate due only to the absence of the symbiont or because of the insect's sensitivity to high temperatures. It could also be because of the low percentage of insects that hatched at 30ºC, and with consequent use of one insect in each instar for the detection of the symbiont coincidently affected the results at 30ºC of the second generation.

Moreover, it is important to add that we used a rich diet to feed *N. viridula* and *A. hilare* which consisted of green beans and peanuts (Todd 1989). On the other hand, for *M. histrionica* we used broccoli that because of its high water content requires *M. histrionica* to spend more time ingesting the diluted nutrients and could have influenced the results (Prado et al. unpublished, Todd 1989, Zahn at al. 2008). In addition, the lower proportion of *M. histrionica* harboring the symbionts at 25ºC (around 45%) during the second generation did not influence its mortality, as showed in chapter 5. However, it may have affected the biological parameters R_0 and *GRR* generated for the second generation. Further demographic studies should compare the effect of high temperature on the development of pentatomid insects reared in different diets.

Earlier studies showed that development time of pentatomids decreases when reared in high temperatures (Vivan and Panizzi 2005, Prado at al. 2006). For example *N. viridula* developed more rapidly at higher temperatures (Prado et al. 2006). Here, our data showed that *A. hilare* and *M. histrionica* nymphal development time was not fastened by the increase in temperature; however *A. hilare*'s development time was longer in the absence of its gut-associated symbiont (Prado et al. unpublished). Abe et al (1995) showed that *P. stali*'s development is affected when its egg masses are submitted to surface sterilization; however others showed that surface sterilization of egg masses had no impact on the development of *N. viridula* (Hirose et al. 2006, Lockwood and Story 1986, Prado et al. 2006, Prado et al. unpublished). These results suggest that both higher temperature and symbiont absence could have equalized the development time of the insects from 25ºC and 30ºC at least for *A. hilare*. Furthermore, the data suggests that *A. hilare*'s and probably *M. histrionica*'s symbiont may play an important role in development of the insects. Clearly, further work considering other abiotic factors on the insect's development such as photoperiod should be done.

This study emphasized the importance of temperature in symbiotic relationships between pentatomids and their gut-associated bacteria and adds information to the limited knowledge on the effects of temperature on the maintenance and biology of two economic important species of stink bugs. These results also suggest that probably absence of symbionts, instead of temperature, is affecting the development of the insects and that high temperature can be used as a good method for sterilization of symbionts from the insect's gut. In addition, high temperatures also cause the population of *B. aphidicola*, the primary symbiont of aphids, to decrease and caused the aphid's infertility, which can be compensated by facultative symbionts (Montllor et al. 2002, Koga et al. 2003, Russell and Moran 2006). Dunbar et al. (2007) showed that some populations of *Buchnera* that occur in aphids populations subjected to high temperatures have a mutation in their promoter of a heat shock gene. This gene permits increased aphids reproduction when they were exposed to high temperature compared to controls. This single base-pair mutation increases the aphid's ability to withstand high temperatures, which appears to have an important role in the aphid's ability to colonize warmer regions (Dunbar at al. 2007). Additionally, it is been shown that environmental temperature changes and consequent global warming is causing a variety of changes in insect responses such as distribution range, abundance, voltinism and phenology in heteropterans (Musolin 2007). More research on the ecological importance of rise in temperature of many habitats globally on symbiont-dependent insects is necessary. Furthermore, other insects that depend on endosymbionts may be influenced by environmental temperature changes.

6.5 TABLES

Table 6.5.1. Median development time of *Acrosternum hilare* and *Murgantia histrionica* at 25ºC and 30ºC over two generations.

Species	Experiment	Stage	Temperature/Generation			
			25˚C/G1 (n[1])	25˚C/G2 (n)	30˚C/G1 (n)	30˚C/G2 (n)
Acrosternum hilare	I	1st instar	11.36 ± 0.54 (14)	-	9.13±0.15 (11)	-
		2nd instar	17.43 ± 0.62 (14)	-	14.86±0.27 (11)	-
		3rd instar	23.21 ± 0.63 (14)	-	22.06±0.64 (11)	-
		4th instar	29.37 ± 0.63 (14)	-	31.83±0.52 (11)	-
		5th instar	39.99 ± 0.46 (13)	-	45.67±1.43 (11)	-
	II	1st instar	9.97 ± 0.61 (14)	14.61±1.42 (9)	8.33±0.21 (12)	9.50 (7)
		2nd instar	17.12 ± 0.58 (14)	21.88±1.34 (9)	15.58±0.34 (12)	18.64±1.08 (7)
		3rd instar	23.59 ± 1.17 (14)	28.89±1.31 (9)	21.42±0.83 (12)	25.35±1.33 (7)
		4th instar	30.12 ± 1.35 (14)	34.75±1.82 (6)	28.20±1.24 (8)	32.50±5.00 (2)
		5th instar	39.87 ± 1.17 (14)	41.17±1.89 (6)	38.10±1.50 (7)	41.00±3.50 (2)
Murgantia histrionica	III	1st instar	12.16 ± 0.19 (16)	10.82±0.20 (42)	9.16±0.27 (16)	8.20±0.42 (14)
		2nd instar	19.84 ± 0.27 (16)	19.21±0.56 (42)	14.76±0.22 (16)	14.66±0.39 (14)
		3rd instar	27.52 ± 0.64 (16)	27.14±0.42(41)	21.27±0.36 (16)	22.81±1.05 (14)
		4th instar	36.51 ± 0.78 (15)	37.51±0.68 (38)	29.22±0.44 (16)	33.76±1.40 (9)
		5th instar	51.85 ± 1.43 (13)	53.24±0.73 (36)	41.78±0.93 (14)	47.99±1.50 (2)

[1]Number of replicates analyzed.

Table 6.5.2. Effect of temperature on the biological parameters of *Acrosternum hilare* and *Murgantia histrionica*.

Species	Temperature/ Generation	n/n[1]	R_0 (♀/♀)[2]	*T* (days)[3]	*r* (♀/♀/ day)[4]	λ (♀/♀/ day)[5]	*DT* (days)[6]	*GRR*[7]
Acrosternum hilare	25˚C/G1	1/33	9.386	99.454	0.023	1.023	30.782	59.15
	30˚C/G1	1/20	0.969	83.654	-0.0004	0.9996	-[8]	21.24
Murgantia histrionica	25˚C/G1	5/8	22.27 ± 4.45a[9]	94.31 ± 13.20a[10]	0.03 ± 0.01a[11]	1.04 ± 0.01a[12]	26.49 ± 9.20a[13]	49.08 ± 7.89a[14]
	25˚C/G2	10/15	11.96 ± 2.68a	80.74 ± 4.24a	0.02 ± 0.003a	1.03 ± 0.003a	34.47 ± 5.80a	20.90 ± 3.28bc
	30˚C/G1	5/9	8.27 ± 2.38a	61.25 ± 1.67a	0.03 ± 0.01a	1.03 ± 0.01a	119.65 ± 100a	40.21 ± 8.66ac
	30˚C/G2	0	-[15]	-	-	-	-	-

[1] Number of replicates analyzed/total number of females by the time of the first oviposition in the replicates.

[2] Net reproductive rate.

[3] Mean generation time.

[4] Instantaneous rate of increase.

[5] Finite rate of increase.

[6] Doubling time.

[7] Gross reproductive rate.

[8] Impossible to calculate.

[9] Means followed by the same letters in the same column are not significantly different ($F = 0.657$; d.f. = 1; $p = 0.428$);

[10] ($F = 0.076$; d.f. = 1; $p = 0.786$);

[11] ($F = 1.452$; d.f. = 1; $p = 0.244$);

[12] ($F = 1.514$; d.f. = 1; $p = 0.234$);

[13] ($F = 0.560$; d.f. = 1; $p = 0.464$);

[14] ($F = 10.877$; d.f. = 1; $p = 0.004$).

[15] no data were produced.

Table 6.5.3. Effect of temperature on oviposition parameters of *Acrosternum hilare* and *Murgantia histrionica*.

Parameters	*Acrosternum hilare*		*Murgantia histrionica*			
	25˚C/G1	30˚C/G1	25˚C/G1	25˚C/G2	30˚C/G1	30˚C/G2
Adult emergence[1]	35	35	42.60±1.44ac[2]	47.50±1.56a	35.20±1.07bc	46.00±2.00ab
Pre-oviposition period	28	14	23.40±3.20a[3]	21.60±2.27a	20.40±1.99a	-[4]

Oviposition period	70	70	56.40±11.98a[5]	16.60±4.21a	17.00±5.43a	-
Total number of egg masses	88	73	13.20±2.56a[6]	5.70±1.07a	6.20±2.24a	-
Number of eggs/ egg mass	16.48	7.75	10.33±0.81a[7]	11.33±0.32a	11.40±0.50a	-

[1]Day that the first adult emerged.

[2] Means followed by different letters in the same row are significantly different (F = 10.686; d.f. = 3; p = 0.0003).

[3] Means followed by the same letters in the same row are not significantly different (F = 0.014; d.f. = 1; p = 0.907).

[4] No data available.

[5] Means followed by the same letters in the same row are not significantly different (F = 4.363; d.f. = 1; p = 0.051);

[6] (F = 2.916; d.f. = 1; p = 0.105);

[7] (F = 4.367; d.f. = 1; p = 0.051).

6.6 FIGURES

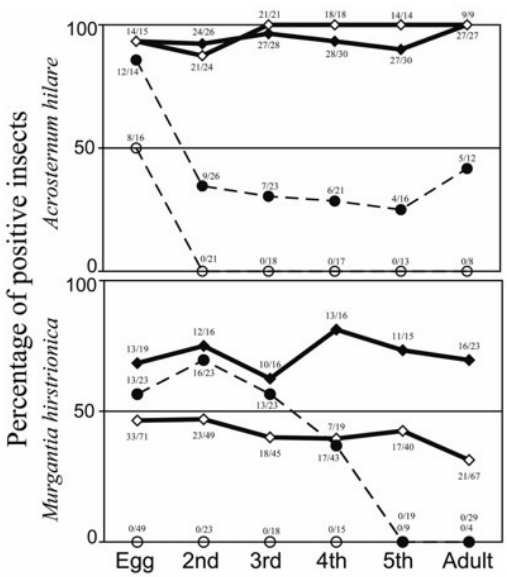


Figure 6.6.1. Percentage of symbiont-positive *Acrosternum hilare* (top) and *Murgantia histrionica* (bottom). Solid lines with black and white diamonds are values at 25ºC for first and second generations, respectively. Dashed lines with black and white circles are values at 30ºC for first and second generations, respectively. Numbers above data points show the proportion of insects infected and the total number of insects tested.

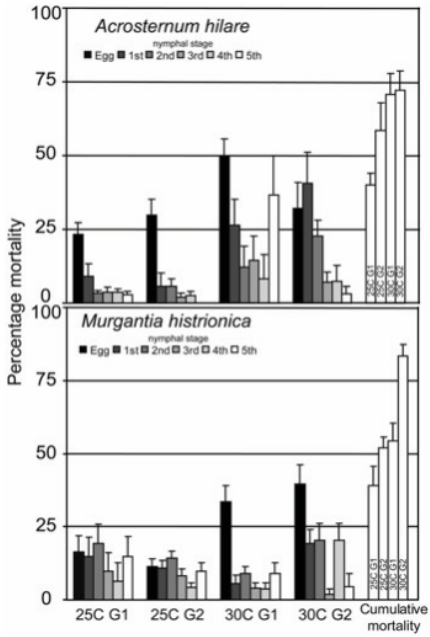


Figure 6.6.2. Percentage of mortality (+SE) in egg viability and 5 nymphal stages of *Acrosternum hilare* (top) and *Murgantia histrionica* (bottom) at 25 and 30ºC during first (G1) and second (G2) generations. White columns on the right represent the cumulative mortality at 25 and 30ºC for the first and second generation.

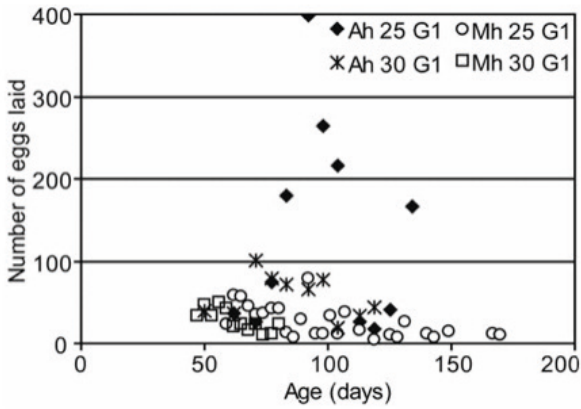


Figure 6.6.3. Total number of eggs laid by *Acrosternum hilare* (Ah) and *Murgantia histrionica* (Mh) cohorts at 25ºC and 30ºC during the first generation (G1).

LITERATURE CITED

Abe, Y., Mishiro K., and M. Takanashi. 1995. Symbiont of brown-winged green bug *Plautia stali* Scott. Jpn. J. Appl. Entomol. Zool. 39: 109-115.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers, New York.

Carey, J.R. 1993. Applied demography for biologists with special emphasis on insects. Oxford University Press, Oxford, England.

Chen, D.Q., Montllor, C.B., and A.H. Purcell. 2000. Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfafa aphid, *A. kondoi*. Entomol. Experim. Appl. 95: 315-323.

Crawley, M.J. 2005. Statistics: An introduction using R. John Wiley & Sons, England.

Dunbar, H.E., Wilson, A.C.C., Ferguson, N.R., and N.A. Moran. 2007. Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. PLoS Biol. 5: 1006-1015.

Durvasula, R.V., Kroger, A., Goodwin, M., Panackal, A., Kruglov, O., Taneja, J., Gumbs, A., Richards, F.F., Beard, C.B. and C. Cordon-Rosales. 1999. Strategy for introduction of foreign genes into field populations of chagas disease vectors. Ann. Entomol. Soc. Amer. 92: 937-943.

Durvasula, R.V., Sundaram, R. K., Cordon-Rosales, C., Pennington, P. and C.B. Beard. 2003. *Rhodnius prolixus* and its symbiont, *Rhodococcus rhodnii*: a model for paratransgenic control of disease transmission. Insect Symbiosis. K. Bourtzis and Miller, T. A., CRC Press: 83-95.

Fukatsu, T. and T. Hosokawa. 2002. Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. Appl. Environ. Microbiol. 68: 389-396.

Hirose, E., Panizzi, A.R., De Souza, J.T., Cattelan A.J., and J.R. Aldrich. 2006. Bacteria in the gut of southern green stink bug (Heteroptera: Pentatomidae). Ann. Entomol. Soc. Am. 99: 91-95.

Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., and T. Fukatsu. 2006. Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. PLoS Biology 4: 1841-1851.

Hosokawa, T., Kikuchi, Y., Shimada, M., and T. Fukatsu. 2007. Obligate symbiont involved in pest status of host insect. Proc. R. Soc. B. 274: 1979-1984.

Kikuchi, Y., Hosokawa, T., and T. Fukatsu. 2007. Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. Appl Environ Microbiol 73: 4308-4316.

Kikuchi, Y., Meng, X.Y., and T. Fukatsu. 2005. Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Hemiptera: Alydidae). Appl Environ Microbiol 71: 4035-4043.

Koga, R., Tsuchida, T., and T. Fukatsu. 2003. Changing partners in an obligate symbiosis: a facultative endosymbiont *Buchnera* in an aphid. Proc. R. Soc. Lond. B. 270: 2543-2550.

Lockwood, J.A., and R.N. Story. 1986. Adaptive functions of nymphal aggregation in the southern green stink bug, Nezara viridula (L.) (Hemiptera: Pentatomidae). Environ. Entomol. 15: 739-749.

Montllor, C.B., Maxmen, A., and A.H. Purcell. 2002. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecol. Entomol. 27: 189-195.

Musolin, D.L. 2007. Insects in a warmer world: ecological, physiological and life-history responses of true bugs (Heteroptera) to climate change. Global change biol. 13: 1565-1585.

Ohtaka, C., and H. Ishikawa. 1991. Effects of heat treatment on the symbiotic system of an aphid mycetocyte. Symbiosis 11: 19-30.

Peterson, W.T., and S.J. Painting. 1990. Developmental rates of the copepods *Calanus australis* and *Calanoides carinatus* in the laboratory, with discussion of methods used for calculation of development time. J. Plankton Res. 12: 283-293.

Prado, S.S., and R.P.P., Almeida. 2008. Phylogenetic placement of pentatomid stink bug gut symbionts. Curr. Microbil. Accepted.

Prado, S.S., Rubinoff, D., and R.P.P. Almeida. 2006. Vertical transmission of a pentatomid caeca-associated symbiont. Ann. Entomol. Soc. Am. 99: 577-585.

R Development Core Team 2006 R: a language and environment for statistical computing. Vienna, Austria: R Foundation for statistical computing. See http://www.R-project.org

Russell J.A., and N.A. Moran. 2006. Cost and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proc. R. Soc. Lond. B. 273: 603-610.

Todd, J.W. 1989. Ecology and behavior of *Nezara viridula*. Ann. Rev. Entomol. 34: 273-292.

Vivan L.M., and A.R. Panizzi. 2005. Nymphal and adult performance of genetically determined types of *Nezara viridula* (L.) (Heteroptera: Pentatomidae), under different temperature and photoperiodic conditions. Neotrop. Entomol. 34: 911-915.

Zahn, D.K., Girling, R.D., McElfresh, J.S., Carde, R.T., and J.G. Millar. 2008. Biology and reproductive behavior of *Murgantia histrionica* (Heteroptera: Pentatomidae). Ann. Entomol. Soc. Am. 101: 215-228.

APPENDIX

APPENDIX A

Proportion of symbiont positive individuals of *Acrosternum hilare* for two generations tested by PCR.

Stage	Treatment/Generation			
	Control/G1	Control/G2	SS[1]/G1	SS/G2
Egg masses	16/16	20/20	15/17	18/20
Nymphs	-[2]	20/20	-	15/19
Adults	5/5	35/35	0/5	35/35
Total	21/21a[3]	75/75a	15/22a	68/74a

[1] SS = Surface sterilized.

[2] Not sampled.

[3] Proportion followed by same letters in the same row are not significantly different ($\chi^2 = 1.124$, d.f. = 3, $p = 0.77$).

APPENDIX B

Stage and cumulative percentage of mortality (+SE) in 5 nymphal stages of *Acrosternum hilare* at control and surface sterilized (SS) egg masses during first (G1) and second (G2) generations.

Treatments/ Generation	n[1]	1st instar	2nd instar	3rd instar	4th instar	5th instar	Cumulative Mortality
Control/G1	16	5.91±1.7	14.11±3.7	1.88±0.9	7.79±3.1	4.38±1.8	34.07±6.05a[3]
Control/G2	20	2.32±1.0	4.32±1.6	10.71±2.8	5.89±2.0	12.04±4.7	30.17±5.37a
SS[2]/G1	17	12.59±5.0	48.86±6.0	12.80±2.8	4.92±1.7	4.59±1.8	67.85.±3.68b
SS/G2	19	2.64±0.8	7.31±3.9	10.61±2.6	8.71±4.0	13.39±3.9	39.38±6.57c

[1] Number of cohorts.

[2] SS = Surface sterilized.

[3] Means followed by different letters in the same column are significantly different (interaction between treatment and generation $F = 11.66$, d.f. = 3, $p = 0.001$).

APPENDIX C

Mean nymphal development time (+SE) in the 5 nymphal stages of *Acrosternum hilare* in control and surface sterilized (SS) egg masses at first (G1) and second (G2) generation.

Age	Treatments/Generation			
	Control/G1 (n[1])	Control/G2 (n)	SS[2]/G1 (n)	SS/G2 (n)
Hatched - 1st instar	5.73±0.10[3] (16)	4.98±0.52 (20)	6.46±0.27 (14)	5.91±0.28 (18)
Hatched - 2nd instar	13.10±0.24 (16)	11.50±0.25 (20)	19.37±0.83 (14)	13.93±0.74 (18)
Hatched - 3rd instar	18.02±0.31 (16)	18.06±0.45 (20)	30.18±1.18 (14)	20.11±0.74 (18)
Hatched - 4th instar	24.60±0.36 (16)	26.49±0.35 (20)	38.97±1.38 (14)	27.93±1.00 (18)
Hatched - 5th instar	35.96±0.66a[4] (16)	39.11±0.65a (20)	52.32±1.62b (14)	41.10±1.41b (18)

[1] Number of cohorts.

[2] SS = Surface sterilized.

[3] Data censused every day.

[4] Means followed by different letters in the same row are significantly different (t = -2.87, d.f. = 319, p = 0.0044).

APPENDIX D

Effect of egg mass surface sterilization on demographic parameters of *Acrosternum hilare*.

Treatments/Generation	n[1]	R_0 (♀/♀)[2]	T (days)[3]	r (♀/♀/day)[4]	λ (♀/♀/day)[5]	DT (days)[6]	GRR[7]
Control/G1	193	31.212[8]	75.849	0.045	1.046	15.280	129.99
SS[9]/G1	64	5.928	74.464	0.024	1.024	29.003	76.15

[1] Number of adults when start to lay eggs.

[2] $R_0 = \sum l_x m_x$ - net reproductive rate.

[3] $T = (\sum x\, l_x m_x)/R_0$ - mean generation time.

[4] $r = ln(R_0)/T$ - instantaneous rate of increase.

[5] $\lambda = exp\ (r)$ - finite rate of increase.

[6] $DT = ln2/r$ - population doubling time.

[7] $GRR = \sum m_x$ - gross reproductive rate.

[8] Data censused every day.

[9] SS = surface sterilized.

APPENDIX E

Effect of egg mass surface sterilization on oviposition parameters of *Acrosternum hilare*.

Parameters	Treatment	
	Control	SS[1]
Adult emergence[2]	30[3]	33
Pre-oviposition period	20	17
Oviposition period	62	61
Total number of egg masses	194	53
Total number of eggs/egg mass	23	19

[1] SS = Surface sterilized.

[2] Day that the first adult emerged.

[3] Data censused every day.

APPENDIX F

Proportion of symbiont positive individuals for *Acrosternum hilare* in three treatments: entire egg mass, physically broken egg mass and surface sterilized egg mass.

Stage/Generation	Treatments		
	Entire EM[1]	Broken EM[2]	SS EM[3]
EM/G1	5/5	5/5	1/5
Nymphs/G1	8/10	10/10	2/10
Adults/G1	10/12	7/8	0/7
EM/G2	14/14	42/44	0/6
Total G1	23/27a[4]	22/23a	3/22b

[1] EM = egg mass.

[2] Physically broken EM.

[3] SS EM= Surface sterilized EM.

[4] Proportion followed by different letters in the same row are significantly different ($\chi^2 = 40.70$, d.f. = 2, $p < 0.0001$).

APPENDIX G

Stage and cumulative percentage of mortality (+SE) for *Acrosternum hilare* in three treatments: entire, physically broken and surface sterilized egg mass.

Stage Mortality	Treatments		
	Entire EM[1] (n=5)[2]	Broken EM[3] (n=5)	SS EM[4] (n=5)
Hatching	38.32±14.7[5]	22.66±9.1	13.97±6.5
1st instar	45.11±11.4	28.43±12.3	30.31±10.0
2nd instar	25.48±9.8	15.67±6.7	38.09±7.7
3rd instar	0	2.5±2.5	21.00±8.7
4th instar	0	1.43±1.4	20.00±20
5th instar	7.16±4.4	0	0
Cumulative Mortality	76.69±7.51a[6]	56.03±10.17a	74.28±8.50a

[1] EM = egg mass.

[2] Number of cohorts.

[3] Physically broken EM.

[4] SS EM= Surface sterilized EM.

[5] Data censused every five days.

[6] Percentage followed by letters in the same row are not significantly different ($F = 1.65$, d.f. $= 2$, $p = 0.23$).

APPENDIX H

Effect of physical separation of the eggs and surface sterilization of egg mass on demographic parameters of *Acrosternum hilare*.

Treatments	n/n[1]	R_0 (♀/♀)[2]	T (days)[3]	r (♀/♀/day)[4]	λ (♀/♀/day)[5]	GRR[6]
Entire EM[7]	1/7	8.47[8]	96.66	0.02	1.02	85.36
Broken EM[9]	4/10	25.97±5.62a[10]	111.45±2.48a[11]	0.03±0.002a[12]	1.03±0.002a[13]	89±15.77a[14]
SS EM[15]	2/3	0.98±0.02b	172.94±27.94b	0±0.0001b	1±0.0001b	9±4.25b

[1] Number of cohorts used for the calculation of the parameters/total number of females by the time of the first oviposition in the replicates.

[2] $R_0 = \sum l_x m_x$ - net reproductive rate.

[3] $T = (\sum x\, l_x m_x)/R_0$ - mean generation time.

[4] $r = ln(R_0)/T$ - instantaneous rate of increase.

[5] $\lambda = exp\ (r)$ - finite rate of increase.

[6] $GRR = \sum m_x$ - gross reproductive rate.

[7] EM = egg mass.

[8] Data censused every five days.

[9] Physically broken EM.

[10] Means followed by different letters in the same column are significantly different ($F = 8.80$, d.f. = 1, $p = 0.04$);

[11] ($F = 12.33$, d.f. = 1, $p = 0.024$);

[12] ($F = 77.06$, d.f. = 1, $p < 0.001$);

[13] ($F = 8.22$, d.f. = 1, $p = 0.046$);

[14] ($F = 11.23$, d.f. = 1, $p = 0.029$).

[15] SS EM= Surface sterilized EM.

APPENDIX I

Effect of surface sterilization of egg masses on oviposition parameters of *Acrosternum hilare*.

Parameters	Treatment		
	Entire EM[1] (n[2])	Broken EM[3] (n)	SS EM[4] (n)
Adult emergence[5]	50[6] (1)	50±0.85a[7] (4)	89±13.50b (2)
Pre-oviposition period	35 (1)	34±3.15a[8] (4)	78±7.50b (2)
Oviposition period	45 (1)	71±6.25a[9] (4)	15± 10.00b (2)
Number of eggs/egg mass	27 (1)	25±3.76a[10] (4)	8±1.63b (2)

[1] EM = egg mass.

[2] Number of cohorts.

[3] Physically broken EM.

[4] SS EM= Surface sterilized EM.

[5]Day that the first adult emerged.

[6] Data censused every five days.

[7] Means followed by different letters in the same row are significantly different ($F = 20.91$, d.f. = 1, $p = 0.04$);

[8] ($F = 44.14$, d.f. = 1, $p = 0.003$);

[9] ($F = 25.23$, d.f. = 1, $p = 0.007$);

[10] ($F = 8.22$, d.f. = 1, $p = 0.046$).

APPENDIX J

Effect of surface sterilization on oviposition parameters in *Acrosternum hilare* and *Murgantia histrionica*.

Species	Treatment/ Generation	n/n[1]	Adult emergence[2]	Pre-oviposition period	Oviposition period	Number of eggs per egg mass
Acrosternum hilare	Control/G1	11/38	54±1.56[3]	38±4.57	86±25.04	25±2.92
	SS[4]/G1	1/1	77	120	5	8
Murgantia histrionica	Control/G1	8/16	67±1.68[5]a[6]	27±3.4a[7]	44±12.01a[8]	11±0.22a[9]
	Control/G2	4/5	75±5.56a	54±7.74b	26±4.27a	9±1.04a
	SS/G1	6/14	69±1.28a	33±4.03a	53±14.93a	10±0.56a
	SS/G2	0/0	-[10]	-	-	-

[1] Number of cohorts used for the calculation of the parameters/total number of females by the time of the first oviposition in the replicates.

[2]Day that the first adult emerged.

[3] Values of *A. hilare* = Median ± confidence interval (95% CI, n = 11).

[4] SS = surface sterilized.

[5] Values of *M. histrionica* = Median ± standard error (SE).

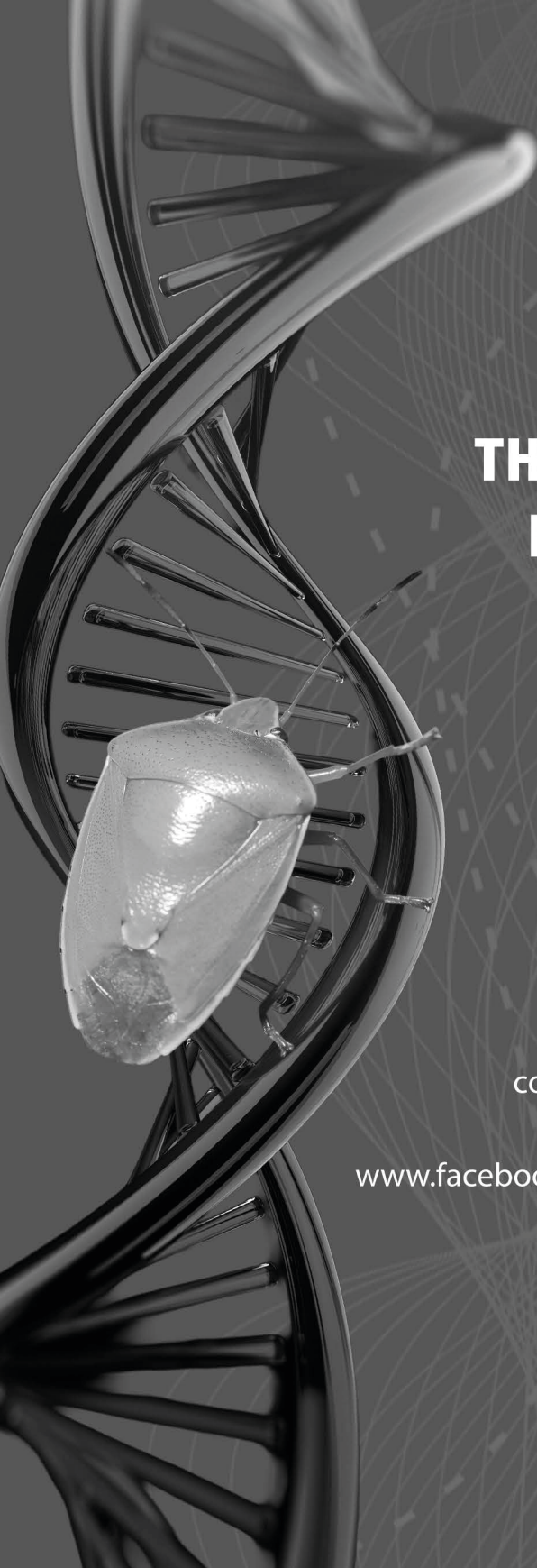
[6] Means followed by same letters in the same column are not significantly different ($F = 2.02$, d.f. = 2, $p = 0.167$).

[7] Means followed by different letters in the same column are significantly different ($F = 7.98$, d.f. = 2, $p = 0.004$).

[8] Means followed by the same letters in the same column are not significantly different ($F = 0.84$, d.f. = 2, $p = 0.451$).

[9] Means followed by the same letters in the same column are not significantly different ($F = 2.71$, d.f. = 2, $p = 0.099$).

[10] No data available.



FOUNDATIONS OF THE MOLECULAR AND BIOLOGICAL STUDY OF SYMBIONTS IN PENTATOMIDAE (HEMIPTERA: HETEROPTERA)

www.atenaeditora.com.br
contato@atenaeditora.com.br
@atenaeditora
www.facebook.com/atenaeditora.com.br

Atena Editora
Year 2026

FOUNDATIONS OF THE MOLECULAR AND BIOLOGICAL STUDY OF SYMBIONTS IN PENTATOMIDAE (HEMIPTERA: HETEROPTERA)

www.atenaeditora.com.br
contato@atenaeditora.com.br
@atenaeditora
www.facebook.com/atenaeditora.com.br

Atena Editora
Year 2026