THE PREVALENCE, DISTRIBUTION, AND IMPACT OF *NOSEMA MADDOXI* INFECTING THE INVASIVE BROWN MARMORATED STINK BUG

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ABSTRACT

Nosema maddoxi is a newly described microsporidian species that is an entomopathogen of the invasive brown marmorated stink bug (*Halyomorpha halys*) and three native stink bug species in the United States. *Halyomorpha halys* is an agricultural and nuisance pest in the US and has been detected in 44 states. Microsporidian species such as *Paranosema locustae* have been used successfully to control insect pest populations, because they negatively impact their host's fitness. Since *N. maddoxi* was present in the field and was also the main cause for the decline of some *H. halys* lab colonies, it was important to investigate the distribution of this entomopathogen and its impact on this invasive species. One thesis objective focused on the phenology, distribution, infection intensity, and prevalence of *N. maddoxi* infections in *H. halys* populations in the US. This study determined that the prevalence of *N. maddoxi* infection was seasonal, with the highest infection levels occurring in the spring and the lowest in the summer. Prevalence of *N. maddoxi* infection was also variable among sites. In addition, *N. maddoxi* infections were widely distributed in established *H. halys* populations in the US as this pathogen was found in CA, KY, MD, NC, NY, OH, OR, PA, UT, VA, and WV; this included all of the states where *H. halys* was sampled for this study. The prevalence of *N. maddoxi* infection was not found to be host density-dependent and low-intensity infections were more common than high-intensity infections in field-sampled populations. Melanized tissues that looked like brown spots were visible through the abdominal cuticle of many infected *H. halys* and this was identified as a potential indicator of *N. maddoxi* infection in

H. halys. For the second thesis objective, bioassays were conducted to investigate the effects of *N. maddoxi* infection on the survival of *H. halys* adult females and nymphs, and on female fecundity, egg viability, and nymphal development. There were two adult female bioassays: 1) treated adult females, in a low spore concentration bioassay, in which each female imbibed 25 µl containing 350,000 spores for 2 replicates and 400,000 spores for one replicate, and 2) treated females, in a high spore concentration bioassay, in which each female imbibed 25 µl containing 875,000 spores. Treated second instar nymphs in the nymphal bioassay each imbibed 2 µl containing 28,000 spores. Results determined that *N. maddoxi* infection significantly shortened the lives of infected nymphs and *H. halys* adult females at the higher spore concentration. In addition, *N. maddoxi* infection negatively impacted female fecundity and egg viability for both spore concentrations. Nymphal development rate and size was not impacted by *N. maddoxi* infection. In summary, these studies determined that *N. maddoxi* is widely distributed in *H. halys* populations, can have high infection prevalence, and shortens the lifespans of *H. halys* adult females and nymphs, as well as decreasing reproduction, indicating that this newly described microsporidian species is a natural biological control agent of this invasive species in the US.

BIOGRAPHICAL SKETCH

Carrie Preston was born on the 6th of April 1992, in Rochester, NY to Jay and Diana Preston. She graduated from high school at Dansville Senior High in 2010. In 2012, she completed her Associate of Science in Environmental Sciences at Finger Lakes Community College where she gained an appreciation of invasive species and conservation management. In 2015, she received her Bachelor of Science in Zoology at SUNY Oswego where her mind became set on becoming a wetland ecologist. This changed after she worked at the USDA APHIS PPQ Otis laboratory in Buzzards Bay, MA, under the supervision of Dr. Hannah Nadel. In this position, Carrie's fascination with insects grew, and after working on several projects such as studying the larval behaviors of the European grapevine moth (*Lobesia botrana*) in a mass-rearing setting and setting up traps to capture emerald ash borer (*Agrilus planipennis*), she decided to become an entomologist, with a focus on invasive species and biological control.

In 2017, Carrie joined Dr. Ann Hajek's invertebrate pathology lab and Dr. Arthur Agnello's tree fruit extension entomology program at Cornell University. Her M.S. project focused on *N. maddoxi*, a newly described microsporidian species of the invasive brown marmorated stink bug (*Halyomorpha halys*). Following graduation, Carrie plans on continuing her education in Forest Entomology, with a focus on invasive species and biological control, involving extension and outreach activities.

Dedicated to Tyler, my family, and friends for their support that has provided me the strength and the determination to pursue my dreams.

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CHAPTER I

The Invasive *Halyomorpha halys* Infected by the Microsporidian *Nosema maddoxi*

1.1 Abstract

Nosema maddoxi Becnel, Solter, Hajek, Huang, Sanscrainte, & Estep is a newly described microsporidian species that infects the brown marmorated stink bug (*Halyomorpha halys* (Stål)). Several microsporidian species have been identified as biological control agents attacking insect pests, but they are difficult to study and to mass produce for augmentative release. *Halyomorpha halys* has caused significant agricultural damage in the Mid-Atlantic region of the US, and several management strategies have been developed in an attempt to control this pest. This review focuses on information gathered on *H. halys*, such as its introduction into the United States and the development of methods for its control. It also focuses on information about microsporidian infections in insects, and the significance of *N. maddoxi* in *H. halys* lab colonies, as well as the potential of *N. maddoxi* as a biological control agent for control of *H. halys* in the field.

1.2 Introduction of *H. halys* into the United States

The brown marmorated stink bug (*Halyomorpha halys* (Stål)) is in the Order Hemiptera, Family Pentatomidae. This species is originally from Korea, Japan, China, and Taiwan (Zhu et al. 2012). In its native range, *H. halys* has been identified as an agricultural pest that feeds on tree fruits such as apple, peach, and cherry, and a variety of field crops such as soybeans and peas (Hoebeke and Carter 2003). *Halyomorpha*

halys has also been reported as a nuisance pest in northern Japan, aggregating in homes and commercial buildings to overwinter (Hoebeke and Carter 2003). A number of natural enemies help control this pest in its native range, including parasitoids such as the samurai wasp (*Trissolcus japonicus* (Ashmead)) (Hymenoptera: Scelionidae), predators such as *Isyndus obscurus* (Dallas) (Hemiptera: Reduviidae), and entomopathogens such as *Ophiocordyceps nutans* (Patouillard) Sung, Sung, Hywel-Jones & Spatafora (Hypocreales: Ophiocordycipitaceae) (Lee et al. 2013).

Northeastern, western, and midwestern states in the United States share climatic conditions similar to those of *H. halys*' native home range (Zhu et al. 2012) and this is consistent with the establishment and spread of *H. halys* in the US for 23 years. *Halyomorpha halys* was first detected in the US in Allentown, PA, in 1996 (Rice et al. 2014). How *H. halys* was first introduced is not known, but it has been intercepted at several entry points in the US and Canada numerous times (Leskey and Nielsen 2018). Early in the invasion *H. halys* had spread from Pennsylvania to states along the East Coast, especially in the Mid-Atlantic region, and small populations were found in southern and western states. Large populations of *H. halys* developed in several eastern states especially around 2010, and populations in the Midwest and on the West Coast are on the rise (Leskey and Nielsen 2018). By 2018, *H. halys* had been detected in 44 states and 4 Canadian provinces in North America (Northeastern IPM Center 2018a).

Halyomorpha halys has also become an invasive pest on other continents, reaching South America and several countries in Europe (Lee 2015). It was first found

in Switzerland in 2007 and since then has been reported in Germany, France, Italy, Greece, Hungary, Spain, and Chile (Leskey and Nielsen 2018). In 2016, the first *H. halys* outbreak populations in Russia, Georgia, and Abkhazia occurred (Gapon 2016).

One study determined that the *H. halys* population introduced to the US originated from Beijing, China (Xu et al. 2014). Only two mtDNA haplotypes were found in *H. halys* populations in eastern states and on the West Coast of the US, whereas 43 mtDNA haplotypes were found in native *H. halys* populations, indicating that the genetic diversity of *H. halys* populations in the US is very low. Based on the results of this study it was assumed that *H. halys* was introduced only once into the US and populations found in California and the inland states were introduced by human transportation from East Coast populations (Xu et al. 2014). However, a later study using a larger sample size found that there were at least three *H. halys* introductions into the US, with one introduction in the eastern US and multiple introductions in the western US (Valentin et al. 2017). This study agreed with the finding by the Xu et al. (2014) study, that there was very low genetic diversity in eastern US *H. halys* populations, but, due to the larger sample size, the Valentin et al. study (2017) found more genetic variation with three additional haplotypes in the western US *H. halys* populations and these had not been found in the Xu et al. (2014) study (Valentin et al. 2017). Both studies confirmed that the introductions were from China. This knowledge can be used to help determine locations to search for natural enemies that could be useful biological control agents for *H. halys* in the US (Xu et al 2014) and to help locate where *H. halys* are being exported from in Asia (Valentin et al. 2017).

1.3 Problems Caused by *H. halys*

In the US, where populations have been established, *H. halys* has been identified as a severe agricultural and nuisance pest, particularly in the Northeast (Leskey and Nielsen 2018). *Halyomorpha halys* is polyphagous, feeding on more than 170 host plants, including tree fruit, row crops, vegetables, and ornamental plants (Northeastern IPM Center 2018b). Reproductive structures of host plants are mainly targeted as food sources by *H. halys* adults and nymphs (Rice et al. 2014). Four to six generations per year occur in Southern China, but in the US, one to two generations occur per year (Nielsen and Hamilton 2009, Rice et al. 2014). This has not limited *H. halys* population growth, and in 2010 the Mid-Atlantic region suffered from *H. halys* population outbreaks that were devastating to agriculture throughout the region (Leskey et al. 2012).

In 2010, *H. halys* caused a loss of $37 million for apple growers in the Mid-Atlantic region (Leskey and Nielsen 2018). During the same year, stone fruit growers lost more than 90% of their crops (Lee 2015). In 2011, at research farms and on-farm plots in Beltsville, Maryland, nearly 100% of sweet corn ears (*Zea mays* L.) were damaged by *H. halys*, and snap bean pods (*Phaseolus vulgaris* L.) in Maryland and Virginia research plots suffered 10-15% damage (Kuhar et al. 2012). Losses caused by *H. halys* in the western and eastern states are presently not as devastating as in the Mid-Atlantic in 2010, but as populations rise, it is predicted that increased damage will occur that will lead to losses in yield and quality of tree fruit, row crops, field crops, and ornamental crops produced in those regions (Leskey and Nielsen 2018).

Current losses due to *H. halys* in specialty, field, and row crops in the Mid-Atlantic region are unknown (Leskey and Nielsen 2018).

The wine industry in the US has also been negatively affected, not only by *H. halys* feeding damage, but also by the presence of the insects at harvest and during the wine-making process (Mohekar et al. 2017). When an *H. halys* individual is disturbed, it releases the stress compounds tridecane and (E)-2-decenal. So, if *H. halys* that are on grape clusters at harvest become disturbed and their bodies end up in the wine production, these compounds are included in the wine, decreasing the finished wine quality (Mohekar et al. 2017). New York State is one of the top grape producers in the US, and this industry could be under threat if *H. halys* populations continue to rise in New York (Smith et al. 2014).

During late summer and autumn (August-October) *H. halys* adults aggregate in large groups to overwinter (Rice et al. 2014). Residential and business structures have become preferred over natural areas for overwintering individuals (Inkley 2012). As a result, *H. halys* has become a well-known nuisance pest wherever it has dispersed or has been introduced (Leskey and Nielsen 2018). In areas where *H. halys* populations are high, massive numbers have been found inside peoples' homes. In Maryland, 26,205 *H. halys* adults were collected using a household vacuum from one house over a 181-day period (Inkley 2012). *Halyomorpha halys* overwintering in the home left stains on walls and curtains from their excrement, and they also produced their characteristic strong odor when stressed. Residents were also disturbed by *H. halys* adults that were attracted to light fixtures at night and these stink bugs were found by homeowners in their beds. Control efforts to prevent future invasions of the home

consisted of caulking potential interior entry points. Daily collections of *H. halys* decreased as major entrances were blocked, but large numbers of *H. halys* continued to infest the home. The highest numbers of *H. halys* found were in the attic crawl spaces, followed by the first floor, second floor and the attic living space. Entryways that were observed during the *H. halys* infestation included bugs crawling behind vinyl siding at corner joints, behind shingle siding, through cracks around ceiling molding, around baseboards, baseboard heaters, floorboards, windows, and light fixtures (Inkley 2012).

1.4 *Halyomorpha halys* Management Strategies

As evidence of the success of this invasive pest, at some locations and times *H. halys* populations have reached uncontrollable levels. Management strategies need to be developed to control population outbreaks and to decrease the amount of agricultural damage this species is causing. To determine the extent to which *H. halys* populations were growing, researchers developed monitoring strategies that involved the use of traps baited with an aggregation pheromone. The original attractant used was the aggregation pheromone of the brown-winged green bug (*Plautia stali* Scott) (Hemiptera: Pentatomidae) (Khrimian et al. 2014). This pheromone was effective in attracting *H. halys* adults and nymphs, but only when the adults were preparing to overwinter (Leskey et al. 2012). After determination of the aggregation pheromone produced by *H. halys* males (Khrimian et al. 2014) the lures produced were more successful in attracting *H. halys* adults and nymphs throughout the summer and fall seasons (Leskey and Nielsen 2018). Monitoring tools baited with or without these

lures that have been used include sticky card panels, UV black light traps, tree canopy-deployed traps (funnel traps, baffle traps, bucket-style water traps, smaller pyramid traps) and ground-deployed traps like the black pyramid trap. In addition, beat sheeting is also used as a monitoring tool, but without the use of pheromones. Black pyramid traps baited with the *H. halys* male aggregation pheromone have proved to be one of the most successful tools for monitoring *H. halys* and have enabled researchers to estimate population sizes in specific areas. These tools assist growers by indicating when *H. halys* is present in their crop, how large the population may be in the area, and when insecticides should be applied (Leskey and Nielsen 2018).

Growers initially had very limited options for protecting their crops from growing *H. halys* populations. The most common approach to control *H. halys* populations, soon after outbreaks first occurred, was the aggressive application of insecticides such as broad-spectrum pyrethroids (Leskey and Nielsen 2018). In one study, insecticides caused a maximum of 60% mortality of overwintering adults, but this decreased to 40% for August applications and 20% mortality for September applications (Leskey et al. 2014). Efficiency of insecticides reached 100% when *H. halys* adults were exposed to fresh insecticide residues, but the mortality rate dropped significantly 3 and 7 days after application. Therefore, effective control of *H. halys* populations would require that insecticides be re-applied every 14 days, resulting in negative ecological consequences. Due to the low impact of insecticide sprays against *H. halys* in their crops, growers have needed to apply four times the amount of sprays they had been using before *H. halys* was present (Leskey et al.

2014). Not only does this harm beneficial insects, such as natural enemies and pollinators, it also causes secondary pest outbreaks (Leskey and Nielsen 2018).

To have fresh residues of sprayed insecticides present for longer time spans in orchards, tree fruit growers tried pesticide applications in alternate rows every 7 days (Leskey and Nielsen 2018). This decreased the sublethal exposure that allowed *H. halys* to recover and increased *H. halys* mortality. *Halyomorpha halys* damage in peppers and tomatoes decreased when a soil-drench application of systemic neonicotinoid insecticides was used, but this was not a registered use in tree fruit (Leskey and Nielsen 2018).

Organic growers were even more restricted in what they could use to protect their crops. Allowed products included organically-approved insecticides, biopesticides, and kaolin clay (Lee et al. 2014). Organically-approved insecticides (azadirachtin, potassium salts of fatty acids, spinosad, pyrethrins, and pyrethrins with kaolin clay) and biopesticides (*Chromobacterium subtsugae* strain PRAA4-1T [MBI-203], extract of *Eucalyptus sp.* [MBI-205], and *Burkholderia sp.* [MBI-206]) were tested on overwintered *H. halys* adults in the lab (Lee et al. 2014). This study determined that all organically-approved insecticides, except azadirachtin, and all biopesticides caused significantly greater mortality than the control over a seven day period. In addition, ≥ 80% of *H. halys* adults were moribund or dead after seven days when pyrethrins with kaolin clay, MBI-203, or MBI-206 were applied, with no significant recovery from the moribund state (Lee et al. 2014). Organically-approved insecticides (insecticidal soap, neem oil, essential oils, and capsaicin) were found to be ineffective against *H. halys* adults, but they did have an impact on nymphs, killing

more than 60% in laboratory trials (Leskey and Nielsen 2018). However, in the field, none of the organically-approved insecticides (azadirachtin, insecticidal soaps, potassium salts of fatty acids, spinosad, pyrethrins, and pyrethrins with kaolin clay) or biopesticide MBI-206 were effective against *H. halys* adults in pepper (Dively et al. 2013).

Habitat manipulation such as trap cropping is a cultural strategy that has been studied in order to manage *H. halys* in organic crop systems (Nielsen et al. 2016). One study determined that the combination of sorghum (*Sorghum bicolor* L.) and sunflower (*Helianthus annuus* L.) was the most effective trap crop out of five trap crops that were tested (sorghum, admiral pea (*Pisum sativum subsp. arvense* (L.)), millet (*Pennisetum glaucum* (L.)), okra (*Abelmoschus esculentus* Moench), and sunflower). But, when these trap crops were evaluated in organic pepper crops at 11 sites in 8 Mid-Atlantic and southeastern states, the reduction in pepper damage was insufficient to be economically viable (Mathews et al. 2017). Additional research on the arrangement of trap crops as well as integrating trap cropping with other management strategies is needed to improve the management of *H. halys* in organic crop systems.

The "attract and kill" strategy that has been used in other systems to control insect pest species was also tested as a management strategy for *H. halys* (Leskey and Nielsen 2018). Field studies indicated that the highest densities of *H. halys* were found along the forest edges bordering both orchards and field crops. In orchards, border trees were baited with the *H. halys* male pheromone and the *P. stali* pheromone to attract *H. halys*. Insecticides were then sprayed to kill *H. halys* that were attracted to

baited trees. Fruit injury was significantly decreased inside the orchard, an indicator that the 'attract and kill' method could be used by growers to protect their crops. This method has not been tested for crops besides fruit (Leskey and Nielsen 2018). Another strategy that has been used was Integrated Pest Management-Crop Perimeter Restructuring (IPM-CPR) in commercial peach orchards in New Jersey (Blaauw et al. 2014). IPM-CPR allowed growers to spray the perimeter of their crop with insecticide but left the majority of the crop unsprayed. This proved to be beneficial and reduced the amount of insecticide used by 25-61%, allowing natural enemies and pollinators to survive while decreasing the amount of damage caused by *H. halys* (Blaauw et al. 2014).

Since the 2010 *H. halys* outbreak, *H. halys* populations in the Mid-Atlantic states remain, and populations are starting to become more established farther north and in the west (Leskey and Nielsen 2018). The negative impacts such as secondary pest outbreaks caused by the insecticides being used to combat *H. halys* populations indicated that a different approach was needed for control; the tactic proposed was classical biological control. In the US, there are several native predators and parasitoids that control native stink bug species, e.g., *Telenomus podisi* Ashmead and *Trissolcus* spp. (Hymenoptera: Scelionidae), spiders (Araneae: Salticidae), earwigs (Dermaptera: Forficulidae), and katydids (Orthoptera: Tettigoniidae) (Abram et al. 2017; Morrison et al. 2018). Studies have shown that native US predators and parasitic wasps are not sufficiently able to reduce *H. halys* populations (Morrison et al. 2018). However, an exotic parasitic wasp *Trissolcus japonicus* (Ashmead) a natural enemy of *H. halys* in Asia, is very successful in controlling *H. halys* populations in its native

range (Morrison et al. 2018). Furthermore, this parasitoid is capable of surviving in climates like the northeastern US (Nystrom Santacruz et al. 2017). Recently, adventive populations of this egg parasitoid were detected parasitizing *H. halys* egg masses in several states in the US, but it has not been detected in all of the locations where *H. halys* has become established (Morrison et al. 2018). This parasitoid appears to have been introduced accidentally to the US (Morrison et al. 2018). In New York, after adventive *T. japonicus* populations were found in 2016, *T. japonicus* was permitted by the Department of Environmental Conservation to be reared and released throughout the state (Jentsch 2017). Since 2017, *T. japonicus* has been released at 32 sites in New York to increase its distribution in the state as well as increasing populations where it has been established (Jentsch 2017). This wasp parasitizes only the egg stage of *H. halys*. Therefore, other biological control methods are needed to control other life stages of *H. halys*.

Entomopathogenic fungi can potentially be biological control agents. In Japan, *O. nutans*, an ascomycete fungus, was found to infect *H. halys* and *P. stali* (Sasaki et al. 2012). This entomopathogenic fungus is naturally occurring in Asia (Japan, China, Korea, Nepal, New Guinea, Taiwan, and India) and Costa Rica, and is not thought to be native to North America. This species parasitizes as many as 22 stink bug species that are considered agricultural and forest pests (Sasaki et al. 2012, Karun and Sridhar 2013). *Ophiocordyceps nutans* attacks the adult stage of stink bugs and, in rare cases, the nymphal stage (Karun and Sridhar 2013). Other entomopathogenic fungi that have been observed parasitizing *H. halys* are *Beauveria bassiana* (Bals.-Criv.) Vuill. and

Metarhizium anisopliae (Metchnikoff) Sorokin (Parker et al. 2015). A commercial product (BotaniGard, BioWorks Inc., Victor, NY) containing the GHA strain of *B. bassiana* was tested on *H. halys* second instar nymphs and adults to determine if it could be used as a biopesticide against *H. halys* (Parker et al. 2015). It was determined that 1 x 10^7 conidia mL^{-1} of the *B. bassiana* GHA strain was able to cause 80%-90% mortality in adults and second instar nymphs after 9 days, and 100% mortality after 12 days (Parker et al. 2015). Nymphs were more susceptible than adults, indicating that BotaniGard could be used to control *H. halys* nymphs when they start to occur in field populations (Parker et al. 2015). However, efficacy trials are still needed to determine the effects of this pathogen strain in different field settings (Parker et al. 2015). *Metarhizium anisopliae* has also been tested and this biopesticide caused lower mortality rates than the GHA strain of *B. bassiana* (Gouli et al. 2012). At a 1 x 10^7 conidia mL^{-1} concentration, *M. anisopliae* caused 40-88% mortality in *H. halys* adults after 12 days. Other life stages of *H. halys* were not tested (Gouli et al. 2012).

Despite all of the different methods available for use by growers to combat *H. halys* populations (i.e., attract and kill, crop perimeter insecticide spraying, biological control, and other IPM tactics) there is still a need for improved control methods. *Halyomorpha halys* populations continue to damage tree fruit and field crops in several Mid-Atlantic states, and have the potential to cause similar damage in western states such as California, Oregon, and Utah (Leskey and Nielsen 2018). More information on the behavior, ecology, and management on *H. halys* is needed to further understand this invasive species, and to decrease its impact on agriculture and as a nuisance pest.

1.5 Investigations of Microsporidia

1.5.1 The New Species Nosema maddoxi

In 1968-1972, Joseph Maddox found a microsporidian species infecting field-collected green stink bugs (*Chinavia hilaris* (Say)) in Illinois (Hajek et al. 2017). He reported this discovery at the 11th annual Society of Invertebrate Pathology meeting in 1978 (Maddox 1979). At that time, this microsporidian species was not officially described, and its effects on its host were unknown (Hajek et al. 2017). In 2012 in Florida and in 2015 in Maryland (AEH pers. comm.) mass-reared *H. halys* colonies were declining. A microsporidian was identified in samples collected from the declining colonies and was determined as the cause of the declines. Comparison of this microsporidian with that found in the samples from Joseph Maddox's 1970-1972 collections showed that it was the same species. This microsporidian species was described as *Nosema maddoxi* Becnel, Solter, Hajek, Huang, Sanscrainte, & Estep *(*Microsporidia: Nosematidae) as it presented characteristics consistent with microsporidia in the genus *Nosema* Nägeli. Evaluations of *H. halys* and native stink bug species collections from 2015-2016 in the Mid-Atlantic region determined that *N. maddoxi* was occurring in field populations of *H. halys*, the green stink bug, the dusky stink bug (*Euschistus tristigmus* (Say)) and the brown stink bug (*E. servus* (Say)) (Hajek et al. 2017).

There are more than 700 microsporidian species that infect insects, often causing negative effects in their hosts (Hoch and Solter 2018). Effects can include a shortened lifespan, reduced fecundity, prolonged larval development, and mortality (Hoch and Solter 2018). A field survey in 2017-2018, found that *N. maddoxi* had a

wide distribution in *H. halys* populations in the US as it was found in 11 states (CA, KY, MD, NC, NY, OH, OR, PA, UT, VA, and WV) (see Chapter 2). In addition, bioassays using adult *H. halys* females and nymphs determined that *N. maddoxi* can shorten the lifespan of adult females and nymphs, negatively impacts female fecundity and egg viability, and causes mortality in nymphal stages (see Chapter 3). Based on these studies and the literature on microsporidia, *N. maddoxi* could provide assistance in controlling *H. halys* populations in the US.

1.5.2 History of Microsporidian Taxonomy

Microsporidia are obligate unicellular parasites, living in the cytoplasm of cells of their hosts (Hoch and Solter 2018). Presently, there are 1300-1500 microsporidian species belonging to approximately 187 genera, described from hosts from all vertebrate orders, several invertebrate orders, and some protists (Hoch and Solter 2018). Ninety-three of the described genera include an insect as a host (Becnel and Andreadis 2014). *Nosema bombycis* Nägeli was the first microsporidian species described; it caused pébrine disease in the silkworm, *Bombyx mori* (L.) (Becnel and Andreadis 2014).

Microsporidia are currently identified as Fungi, but they were not always placed in the Kingdom Fungi (Keeling and Fast 2002). When the first microsporidian species was described, Nägeli (1857) thought *Nosema* was a yeast-like fungus. Based on the morphology of its unique infection apparatus, the polar filament, *Nosema* was eventually separated from the Fungi and later placed within the Sporozoa with other parasite groups. Within Sporozoa, microsporidia were considered to be closely related to myxosporidia and actinomyxidia, which were collectively called the Cnidosporidia

(Keeling and Fast 2002). In 1961, microsporidia were considered to be in the subphylum Cnidospora in the order Microsporidia (Lom and Vávra 1962). In 1980, electron microscopy was a new tool to obtain taxonomic data, and the order Microsporidia in the phylum Microspora was placed as an independent protist lineage (Levine et al. 1980). In 1983, this changed again, and the microsporidia were viewed as evolutionarily significant and identified as one of the four lineages of amitochondriate protists in Archezoa, based on the lack of mitochondria and bacterial-sized rRNA (Keeling and Fast 2002). Molecular phylogenetics eventually supported the position that microsporidia belonged to an ancient and primitive lineage (Keeling and Fast 2002). However, the fact that microsporidia were highly adaptive parasites created doubt about their position on the eukaryotic tree. Phylogenies that contradicted the previous evidence of a primitive lineage were proposed, supporting the proposal that microsporidia were a well-supported group of fungi, based on the presence of alpha-tubulin and beta-tubulin. After determining that microsporidia had a mitochondriate ancestry, the exact relationship of microsporidia with fungi was still hard to decipher. Presently, microsporidia have been identified as highly evolved fungi due to reductive evolution in response to the adaptation to intracellular parasitism (Keeling and Fast 2002).

1.5.3 Microsporidia Life Cycles, Transmission, and Spore Structures

The microsporidian life cycle consists of three main stages: 1. Merogony, the proliferative stage; 2. Sporogony, the spore production stage; and 3. The infective stage, or the mature spore stage (Han and Weiss 2017). Once the sporoplasm is inside the cytoplasm of a host cell, merogony is initiated. During merogony the sporoplasm

develops into multiple meronts by binary fission or multiple fission, depending on the species (Keeling and Fast 2002, Han and Weiss 2017). This results in multinucleate plasmodial forms, which change the shape and size of the host cell (Han and Weiss 2017). Sporogony occurs after merogony. The cell membranes of the meronts thicken, creating sporonts. Sporonts then divide, creating sporoblasts which develop into mature spores. After mature spores have developed the host cell ruptures, releasing a large number of spores that can spread the infection to different cells within the host or they are released into the environment for transmission to new hosts (Keeling and Fast 2002, Han and Weiss 2017).

The life cycles of microsporidia can either be simple or complex, depending on the species (Keeling and Fast 2002). Several species such as *Nosema apis* Zander, and *Vairimorpha necatrix* (Kramer) Pilley complete their life cycles within one host individual (Becnel and Andreadis 2014). Other microsporidian species such as *Amblyospora connecticus* Andreadis and *Edhazardia aedis* (Kudo) Becnel, Sprague, & Fukuda have complex life cycles, where multiple sporulation sequences involve more than one host individual and require more than one generation of the host or an intermediate host (Becnel and Andreadis 2014). *Nosema maddoxi* has a simple life cycle and completes its life cycle within one host individual (Hajek et al. 2017).

Microsporidia are known to be transmitted to their hosts by horizontal transmission (from individual to individual), vertical transmission (from mother to offspring), or both (Hoch and Solter 2018). Horizontal transmission of microsporidia occurs when a non-infected host ingests spores produced from an infected host that had been released into the environment (Becnel and Andreadis 2014, Hoch and Solter

2018). Vertical transmission of microsporidia occurs when an infected female host passes microsporidia to her offspring, either on the inside of the eggs (transovarial) or on the outside of the egg chorion which hatched offspring feed upon (transovum) (Becnel and Andreadis 1999, Goertz et al. 2007). *Nosema maddoxi* spores have been found inside of eggs within the ovaries of infected *H. halys* females although it is not known whether those eggs were viable (Hajek et al. 2017). Newly hatched *H. halys* nymphs aggregate after hatching to feed on excreta from mothers on the egg chorions and thereby obtain symbionts, which promotes their development and survival (Nielsen et al. 2008, Endo and Numata 2017). Therefore, it may be possible that this microsporidian can be vertically transmitted, either transovarially or transovum, but this has not been confirmed.

The microsporidian spore is the key stage for infecting a host and is used to differentiate microsporidian species morphologically (Keeling and Fast 2002). It is the only stage that can survive outside of the host. Most spores are ovoid in shape, but some species are known to have spherical, rod-shaped, or crescent-shaped spores. Some species are differentiated by having several spore types that are produced at different stages in their life cycle. The anatomy of the spore includes two extracellular walls: the exospore wall on the outside, and the endospore wall inside of the exospore wall. Within the spore, the morphology is fairly consistent; sporoplasm, inside the spore membrane, is the infectious material of the spore. There can be a single nucleus or two nuclei, the latter case referred to as a diplokaryon. The polaroplast, the polar filament, and the posterior vacuole are the three main structures required for infection. The polaroplast, a group of membranes in the anterior part of the spore, is separated

into two groups: the lamellar polaroplast and the vesicular polaroplast. The polar filament is the structure used to penetrate the cell membrane of a host cell and to transfer the sporoplasm from the spore into the cytoplasm of the host cell. This structure is attached to the anchoring-disk at the anterior end of the spore and can vary in length from 50-500 µm. Within the spore, the polar filament is straight for one-third of the length of the spore and then is helically coiled within the sporoplasm. The number of coils and the helical tilt of the polar filament are used to identify microsporidian species (Keeling and Fast 2002).

The polar filament (when inside the spore) or polar tube (when discharged out of the spore) is a very complex and highly specialized structure (Han and Weiss 2017). To discharge out of the spore, the polar filament must burst through the anterior pole of the spore located at the anchoring disk. It then everts and uncoils, increasing its length. The polar tube penetrates the cell membrane of a host cell. It is then a hollow tube, which allows the sporoplasm inside the spore to travel through the tube and into the cytoplasm of a host cell. This entire process occurs within two seconds and is believed to occur in several phases that may overlap: 1. Spore activation; 2. An increase in intrasporal osmotic pressure; 3. Eversion of the polar tube; and 4. Passage of the sporoplasm through the everted polar tube and into the host cell's cytoplasm. To discharge and initiate infection (spore germination) certain environmental stimuli must be met. Mechanisms of spore germination remain unclear. The environmental conditions hypothesized as stimulating germination include changes in pH, dehydration followed by rehydration, the presence of certain ions, and the increase of osmotic pressure within the spore. Several microsporidian species have been induced

to germinate in distilled water with 1-5% hydrogen peroxide, but there is no universal stimulant (Han and Weiss 2017).

The genus *Nosema* has more than 150 described species and is found infecting at least 12 insect orders (Becnel and Andreadis 2014). Most *Nosema* species have diplokaryotic developmental stages and spores, but there are a few cases where *Nosema* species have unikaryotic developmental stages and spores (Becnel and Andreadis 2014) and *Nosema maddoxi* is one of the latter (Hajek et al 2017). Molecular data and morphological characteristics of *Nosema maddoxi* support the placement of this species within the *Nosema* genus and in the "true *Nosema* clade", since it has the reversed arrangement of the ribosomal gene LSU-ITS-SSU (Hajek et al. 2017). Species within the genus *Nosema*, not only in the "true *Nosema* clade", are known to cause systemic infections, spreading throughout the tissues of the host, and they can be horizontally and vertically transmitted (Becnel and Andreadis 2014). In agreement, *N. maddoxi* causes systemic infections, with the highest infection levels in the midgut and fat body (Hajek et al. 2017) and this species can be transmitted horizontally (see Chapter 3). *Nosema maddoxi* spores are oblong and measure 4.72 ± 0.05 x 2.19 ± 0.03 µm. Spores have a posterior vacuole with seven-nine polar filament coils and a polaroplast with two regions at the anterior end of the spore (anterior region composed of tightly packed lamellae and the posterior region composed of loosely pack lamellae) (Hajek et al. 2017).

1.5.4 Microsporidian Impacts on Insect Hosts

Microsporidia infections are usually chronic and do not quickly kill their insect hosts (Becnel and Andreadis 2014, Hoch and Solter 2018). However, some

microsporidian species are known to impact the fitness of their insect hosts and can cause a decrease in fecundity, prolong larval development, and a shorter lifespan (Becnel and Andreadis 2014, Hoch and Solter 2018). Several insect pests are impacted by microsporidia, and studies have been conducted in the field and the lab to determine if some microsporidian species could be utilized as biological control agents.

The microsporidian *Nosema fumiferanae postvittana* Hopper, Huang, Solter, & Mills is a pathogen of *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), the light brown apple moth (Hopper and Mills 2016). In the lab, this microsporidian species was horizontally and vertically transmitted (Hopper et al. 2016). Lab results showed a decrease in survival of infected larvae and pupae by 7% and 13%, respectively, 22% reduction in fecundity, 22% decrease in hatch rate, and healthy females lived for five days longer than infected females (Hopper et al. 2016). However, in the field, *N. fumiferanae postvittana* did not have a major impact on the populations of *E. postvittana*. The overall prevalence was 5% and the peak prevalence was 22% in one location in one year. Nevertheless, Hopper and Mills (2016) concluded that this insect pathogen could still be an important population control agent for this invasive insect pest.

Nosema pyrausta (Paillot), a pathogen of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), the European corn borer, is also vertically and horizontally transmitted, and decreases the lifespan and fecundity of its host (Andreadis 1984, 1987). In the field during a two-year study, *N. pyrausta* infection prevalence was found to be enzootic, persisting at low infection levels with 8.3 to 34.0% infection

prevalence in the first year and 2.0 to 17.8% infection prevalence in the second year (Andreadis 1984). In addition, *O. nubilalis* infection prevalence was characterized as "delayed density-dependent", with prevalence being higher in declining *O. nubilalis* populations (Andreadis 1984). It was also determined that in areas where the host larval density was high, the prevalence of *N. pyrausta* infections increased due to the contamination of feeding cavities by the feces of infected larvae (Andreadis 1987). Based on these studies, *N. pyrausta* was determined to be an important naturally occurring biological control agent for *O. nubilalis*.

Paranosema locustae (Canning) Sokolova, Dolgikh, Morzhina, Nassonova, Issi, Terry, Ironside, Smith & Vossbrinck, is an insect pathogen infecting several grasshopper and locust species. It was the first, and is still the only, microsporidian registered and commercially available to growers in the US as a biopesticide (Bjørnson and Oi 2014). This microsporidian can be vertically and horizontally transmitted, but it is believed that vertical transmission is not sufficient for establishing infections in the succeeding year after application. Commercially available spores of *P. locustae* are applied in a bait formulation, and this species is used in fields where damaging grasshopper populations are present. Several studies on efficacy and persistence of *P. locustae* in the field have presented variable results. One study in 1971 reported that after application, the prevalence of *P. locustae* was 43%, grasshopper populations were reduced, and fecundity was also reduced (Henry 1971). Other studies showed inconsistent fecundity reductions, where populations were reduced by 30%, with 20-40% infection prevalence. Although results indicated that this microsporidian was not exhibiting fast and extensive efficacy, it could still

provide assistance in regulating the frequency and severity of grasshopper outbreaks. By utilizing *P. locustae* as a "neoclassical" biological control, countries such as China and Argentina have been able to control native grasshopper species in agroecosystems, when large grasshopper populations were present (Bjørnson and Oi 2014).

Several microsporidian species, whether applied as a biopesticide like *P. locustae*, or naturally occurring like *N. pyrausta*, are considered successful biological control agents. However, there are some hurdles toward further facilitating their use. There are difficulties in mass producing microsporidia for application, not only for use in the field against insect pests but also for study. Unlike some bacteria and other fungi, microsporidia are only produced in living cells and do not reproduce well in tissue culture (Hoch and Solter 2018). However, some microsporidian species have been successfully produced in tissue culture. These include *N. apis*, *N. bombycis*, and *Vavraia culicis* (Weiser) Weiser (Becnel and Andreadis 2014). Production of microsporidia is also very labor-intensive. Investigations of potential target and nontarget organisms involves mass-rearing susceptible hosts, which can be very time-consuming and expensive (Hoch and Solter 2018). Information on the survival of microsporidian spores in the environment after application is lacking, and spore survival has been found to be variable among microsporidian species (Becnel and Andreadis 2014). The only conclusions agreed upon for microsporidian spore survival are: 1. Spores of most species do not survive in the external environment for more than 1 year; 2. Spore survival improves when spores are in feces, dried cadavers, or in an aqueous media at temperatures between 0-6°C; 3. Most microsporidian spores are inviable if at room temperature for two-three months; 4. Spores from aquatic hosts do

not survive being dried; 5. Viability of spores is reduced at temperatures higher than 35°C; 6. Spores cannot survive exposure to direct sunlight for more than several days; and 7. Spore viability can be negatively affected if another microsporidian species is present (Becnel and Andreadis 2014).

Microsporidia also can cause devastating effects to beneficial insects in the field and to insects reared in the lab (Bjørnson and Oi 2014). Colonies of insects reared as biological control agents that are infected are known to have reduced fitness, decreasing their efficacy. Symptoms of a microsporidian infection in the lab include a reduction in food consumption, prolonged larval and pupal development, deformed pupae and adults, a decrease in fecundity and longevity, and increased mortality (Bjørnson and Oi 2014). In the 19th century, silkworm colonies in mass-rearing facilities used for sericulture collapsed due to *N. bombycis,* and this pathogen remains an issue today in sericulture (Becnel and Andreadis 2014). *Ascogaster quadridentata* Wesmael is an endoparasitoid of the codling moth, *Cydia pomonella* (L.) (Bjørnson and Oi 2014). Both the codling moth and this parasitoid are hosts of *Nosema carpocapsae* Paillot. Impacts to *A. quadridentata* parasitizing infected *C. pomonella* include mortality, shortened lifespan, and reduced fecundity. In the 1970s, *Lema cyanella* (L.) was considered as a biological control agent for Canada thistle, *Cirsium arvense* (L.) in Canada. A *Nosema* sp. infected a colony of field-collected *L. cyanella*, which led to the collapse of that colony. It was determined that the *Nosema* sp. infections did not influence adult longevity, but stopped copulation and oviposition and caused high mortality in eggs and larvae. To use *L. cyanella* as a biological

control agent of *C. arvense*, it was recommended that only disease-free *L. cyanella* be released (Bjørnson and Oi 2014).

Once microsporidia are present in a lab colony, it is extremely difficult to remove them and to prevent more infections from occurring (Hoch and Solter 2018). After Louis Pasteur determined that *N. bombycis* was the cause of pébrine disease in silkworms, he created the "Pasteur Technique" (Solter et al. 2012) in which mating pairs are selected and placed in sanitized containers. After oviposition, dissections and microscopy of the mating pairs are used to determine if there are signs of microsporidian infection. Only the offspring of uninfected pairs are reared and if a pair is infected, the offspring are destroyed. Sterilization is also a main component in preventing the spread of microsporidia. All equipment used and areas holding lab colonies are sterilized daily using 5% bleach or another antimicrobial, or by heat. Cadavers of adults and excrement must be separated from eggs, and the eggs should be rinsed either in distilled water, or in 0.25-5.0% bleach if spores are known to be on the egg surfaces (Solter et al. 2012). Sterilizing the egg surfaces is useful for most insect colonies, except for some insects that deposit important symbionts on the surface of eggs for nymphs, such as pentatomids. By following these techniques, labs can avoid and prevent microsporidian infections in colonies of many species of insects (Solter et al. 2012).

1.6 Conclusions

Several management strategies have been developed to combat *H. halys*. To control *H. halys* population outbreaks in the Mid-Atlantic region, growers used heavy

applications of insecticides until improved IPM methods became available. Integrated pest management methods against *H. halys*, such as attract and kill, the use of perimeter insecticide sprays (Integrated Pest Management-Crop Perimeter Restructuring), and the introduction of *T. japonicus* all aided in controlling *H. halys* populations. However, *H. halys* is still spreading, causing crop damage, and acting as a nuisance across the US, and it needs to be controlled. Biological control agents have been found and are being investigated, such as the microsporidian pathogen *N. maddoxi*, a newly described microsporidian. Microsporidia may be difficult to study and to produce, but there have been several studies providing evidence that microsporidia can be an additional asset toward controlling insect pest populations, whether they are applied or naturally occurring. Based on numerous studies to be described in the following chapters, *N. maddoxi* is a naturally occurring biological control agent of *H. halys*.

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CHAPTER 2

Nosema maddoxi (Microsporidia: Nosematidae) in Brown Marmorated Stink Bug (Hemiptera: Pentatomidae) Populations in the US[1]

2.1 Abstract

In 2017, *Nosema maddoxi* Becnel, Solter, Hajek, Huang, Sanscrainte, & Estep (Microsporidia: Nosematidae) was described as a pathogen of the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) (BMSB). In general, microsporidia have been known to negatively impact the fecundity, larval development, and life span of different insect hosts. This study focused on the phenology, prevalence, and distribution of *N. maddoxi* infections in BMSB populations in the United States. Collections of BMSB from three sites in 2018 were evaluated for the seasonality of *N. maddoxi* infections in BMSB populations. Prevalence of infection in spring, after overwintering by BMSB adults, was 37.5 ± 18.9% (peaking at one site at 60.0%) followed by lower infection prevalence during two summer collections (9.7 ± 4.1% and 7.3 ± 2.4%). Collections of BMSB from 31 sites in 11 states in 2017 and 2018 were evaluated and *N. maddoxi* was found in every state sampled, averaging 18.9 ± 4.3% infection (range: 0.0-52.0%). Prevalence of low-intensity infections were more common than high-intensity infections in the

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phenology study (prevalence of low-intensity infection = 69.3%, prevalence of high-intensity infection = 30.7%) as well as in the distribution study (prevalence of low-intensity infection = 62.4%, prevalence of high-intensity infection = 37.6%). There was no association between the prevalence of *N. maddoxi* infection at a site and BMSB population density. Internal melanized tissues within infected BMSB were visible as brown spots through the abdominal cuticle and this physical sign can help indicate *N. maddoxi* infection: 74.2% of BMSB with these symptoms were infected although 30.0% of BMSB adults that were infected did not have spots. Based on this study, this pathogen is widely distributed throughout BMSB populations in the US, the prevalence is variable among sites and is seasonal with the highest infection levels occurring after BMSB adults overwinter.

2.2 Introduction

In 2017, the new species *Nosema maddoxi* Becnel, Solter, Hajek, Huang, Sanscrainte, & Estep was described as a pathogen of the brown marmorated stink bug (*Halyomorpha halys* (Stål)) (BMSB) (Hajek et al. 2017). Field collections of BMSB in 2015-2016 from five eastern states showed variability in the prevalence of *N. maddoxi* infection with a range of 0.0-28.3% in collections from four out of the five states. This species was originally found in native green stink bugs (*Chinavia hilaris* (Say)) collected in Illinois by Joseph Maddox in 1968-1972 (Hajek et al. 2017). Native to Asia, BMSB was first detected in 1996 in Allentown, PA, and was identified as an invasive species in the United States in 2001 (Hoebeke and Carter 2003). Thus, *N. maddoxi* was present in the United States (in IL) several decades before BMSB was

identified as a new invasive species in this country (in PA); *N. maddoxi* is therefore a native entomopathogen infecting an invasive insect (Hajek et al. 2017). *Nosema maddoxi* has also been identified from two other native host species in the United States: the brown stink bug (*Euschistus servus* (Say)) and the dusky stink bug (*Eschistus tristigmus* (Say)) (Hajek et al. 2017).

Microsporidia are known to be transmitted horizontally (host to host), vertically (mother to progeny), or both (Hoch and Solter 2018). Horizontal transmission of microsporidia occurs when a potentially non-infected host ingests microsporidian spores that were left behind in the environment by an infected host (Becnel and Andreadis 2014, Hoch and Solter 2018). Vertical transmission has not been confirmed for *N. maddoxi*, so based on the biology of BMSB, we hypothesize several ways that *N. maddoxi* can be horizontally transmitted. First, BMSB have been observed to feed on unhatched eggs (Endo and Numata 2017), nymphs, freshly eclosed adults, and cadavers (Medal et al. 2012). From these feeding behaviors, non-infected BMSB could ingest *N. maddoxi* spores when they are feeding on infected BMSB. All mobile life stages respond to an aggregation pheromone produced by BMSB males (Khirmian et al. 2014), which is presumably used when a resource such as a host plant, mate, or an overwintering site has been identified (Rice et al. 2014). Therefore, the spread of *N. maddoxi* from an infected BMSB individual to a healthy one (horizontal transmission) could be facilitated by these observed feeding and aggregation behaviors. Another pentatomid has been observed to have similar feeding and aggregating behaviors, and this was shown to lead to the horizontal transmission of a virus (Nakashima et al. 1998).

In the US, BMSB are present year-round, and one to two generations occur per year (Rice et al. 2014). In April through June, BMSB adults disperse from overwintering sites, depending on location (Nielsen et al. 2016). In eastern Pennsylvania, where there is one generation, the first egg masses are laid in mid-June, first instar - third instar nymphs occur in July, and fourth instar - fifth instar nymphs and first-generation adults occur in August (Nielsen and Hamilton 2009). In late summer through the fall, first generation adults aggregate into large groups to overwinter and remain in these groups at an overwintering site throughout the winter until they disperse in the spring (Nielsen and Hamilton 2009). The prevalence of microsporidian species can vary depending on the season in hosts that are present year-round (Hoch and Solter 2018). For example, *Nosema apis* Zander prevalence in *Apis mellifera* L. was highly seasonal and peaked in the spring (Bailey and Ball 1991). Prevalence of *N. apis* was also seasonal in *Apis mellifera scutellata* Lepeletier when *Nosema ceranae* Fries, Feng, Silva, Slemendra, & Pieniazek was not present (Fries et al. 2003). Prevalence of *Amblyospora* sp. infections in *Simulium pertinax* Kollar larvae was highest during the summer months (Araújo-Coutinho et al. 2004). Since BMSB is a host that is present year-round, the prevalence of *N. maddoxi* infections could be seasonal and this could impact BMSB population dynamics.

Depending on the severity of a microsporidian infection, physical signs can occur in infected hosts, including abnormal coloration, and melanized spots visible on the host (Solter et al. 2012a). In the early 1800s, melanized spots that were seen through the larval integument of silkworms (*Bombyx mori* (L.)) were identified as pébrine disease, caused by microsporidia. Melanization of infected cells is a common

immune response to microsporidian infections. These darkened cells can be viewed through the cuticle of an insect, which then is described as having a spotted or mottled appearance (Solter et al. 2012a).

The main objective of this study was to evaluate the temporal and spatial distribution of *N. maddoxi* in BMSB populations in the US, and the intensity of infection in those populations. We predicted that, since *N. maddoxi* is a native microsporidian species, it would be present in BMSB populations where native stink bugs were also present, and that infection intensity and prevalence would vary between seasons and sites, based on field studies on other microsporidian species in insect pest populations (van Frankenhuyzen et al. 2011, Hopper and Mills 2016). Other objectives focused on the association between the prevalence of *N. maddoxi* infection and sex, whether the amount of time BMSB was present at a site correlated with the prevalence of *N. maddoxi* infection, and whether infection prevalence and intensity were associated with BMSB population density or the presence of native stink bugs. We also evaluated whether any physical signs were visible on individual BMSB that indicated *N. maddoxi* infection, as has been observed in other insect host species infected by microsporidia (Tokarev et al. 2006, Hoch and Solter 2018).

2.3 Materials and Methods

2.3.1 Phenology

Three sites sampled in 2017 were chosen to investigate the seasonality of *N. maddoxi* in BMSB populations in 2018. Sites were in New York State and in south-central Pennsylvania (Table 2.1). Four collections of BMSB were made at each site

(one in spring, two in summer, and one in fall). Collections that were sampled from 1 March-30 June were considered spring, collections sampled from 1 July-30 August were considered summer, and fall collections were sampled from 1 September-31 October. Summer collection one was sampled from 1-31 July and summer collection two was sampled from 1-31 August. A total of 709 bugs (59.1 ± 6.4 BMSB/collection (range: 12-100 BMSB) were collected and diagnosed. BMSB were collected near black pyramid and sticky traps baited with aggregation pheromone lures, as well as from the traps themselves. Only overwintering adults were collected during the spring collections and during fall collections 93.1± 4.0% BMSB collected were adults. Summer collection one included 37.5% adults and 62.5% nymphs and summer collection two included 50.6% adults and 49.4% nymphs. Both adults and nymphs were evaluated in this phenology study.

Table 2.1. Collection sites used for the 2018 phenology study.

Site Name	State	County	GIS
Schutt's	NY	Monroe	43.183516, -77.455785
Hudson Valley	NY	Ulster	41.746842, -73.965858
Mickley	PA	Adams	39.956828, -77.304854

2.3.2 Field Survey

In 2017 and 2018, collaborators from 31 sites in 11 states (CA, KY, MD, NC, NY, OH, OR, PA, UT, VA, and WV) collected BMSB specimens, and native stink bugs present when BMSB were collected. Specimens were sampled using black pyramid traps, sticky cards, beating sheets, or by hand. There was a total of 47 collections, with one-four collections made from each site. The mean number of BMSB sampled per site was 67.9 ± 11.9 (range: 2-278) and a total of 2,106 BMSB

were diagnosed at Cornell University. Only adult BMSB were included in this field survey study. All BMSB collected from Pennsylvania (n = 927) were diagnosed and $\leq$ 50 samples were evaluated for all other collection sites. For collection sites with < 50 BMSB, only collections comprising at least 10 BMSB were included in the statistical analyses.

Collection time was categorized based on the season when collections were sampled, as in the phenology study. For a few sites, collections were merged across seasons by collectors and these were only used for distributional analyses.

BMSB population densities at collection sites were estimated based on numbers of the BMSB adults collected in black pyramid traps in the fall (Table 2.2). These BMSB population densities from collection sites (n = 5) were then used to determine if there was an association between *N. maddoxi* overall infection prevalence (the percent of infected hosts in a population) (Poulin 2007) with BMSB population density. BMSB population density was not determined for collection sites that utilized other sampling methods (sticky panel traps, beat sheeting, and hand collecting) and for collection sites that were sampled in the spring or summer.

As part of the analyses, we determined whether the prevalence of *N. maddoxi* infection in a state was associated with the year BMSB was first reported from that state (Hamilton et al. 2018). For this analysis, only eastern sites with collections sampled in 2017 during the fall (n = 12) were included. Only these sites were used because BMSB was introduced once in the East, and multiple introductions could have occurred in the western US (Xu et al. 2014, Valentin et al. 2017).

Table 2.2. Collection sites used in the 2017-2018 field survey to determine the BMSB population density in fall (Sept. – Oct. 2017). BMSB population density was determined by the average number of BMSB adults collected per black pyramid trap when BMSB collections were sampled at a site.

Site #	State	County	GIS	Habitat Type	# of Traps	Collection Date	# BMSB Evaluated	BMSB Population Density Mean (±SE)
3	NY	Monroe	43.183516, -77.455785	Forest edge adjacent to apple orchard	3	9/12/17	15	4.7 (1.3)
3	NY	Monroe	43.183516, -77.455785	Forest edge adjacent to apple orchard	3	9/21/17	50	81.3(60.1)
3	NY	Monroe	43.183516, -77.455785	Forest edge adjacent to apple orchard	3	9/27/17	50	64.0(42.5)
3	NY	Monroe	43.183516, -77.455785	Forest edge adjacent to apple orchard	3	10/4/17	35	27.7(3.3)
4	NY	Ontario	43.261474, -77.369925	Forest edge adjacent to apple orchard	3	9/27/17	38	12.7(9.2)
4	NY	Ontario	43.261474, -77.369925	Forest edge adjacent to apple orchard	3	10/4/17	12	3.7(1.7)
6	NY	Ulster	41.746842, -73.965858	Forest edge adjacent to apple orchard	1	9/26/17	10	54.0
11	OR	Clackamas	44.280065, -122.654692	Edge and inside a hazelnut orchard	6	9/26/17	50	8.0(3.4)
18	VA	Frederick	39.243306, -78.162011	Forest edge adjacent to apple orchard	3	9/12/17	18	2.0(1.3)

2.3.3 Specimen Diagnosis

Throughout this study, microscopy was used to determine whether BMSB specimens were infected with *N. maddoxi* and to determine the intensity of infection (the spore load in an infected host) (Poulin 2007). Specimens of BMSB were stored at -20^{o}C prior to microscopic examination. To determine if an individual was infected with *N. maddoxi,* the specimen was sexed and placed in a 1.5-ml Eppendorf tube with 500 µl of tap water. A micropestle was used to crush the bug eight-ten times and 10 µl of the resulting suspension was viewed at 400X with phase contrast under an 18 mm x 18 mm coverslip. Five fields of view were examined to determine if microsporidian spores were present in the sample. One field of view at 400x measured 0.20 mm^2 and the measurement for five fields of view was 0.98 mm^2. The total area of the 18 mm x 18 mm coverslip was 144 mm^2. To determine the total number of spores per 10 µl of the bug suspension, the total number of spores for the five fields of view was multiplied by the number of times the measurement of the five fields of view fit the total area of the coverslip. This was then multiplied by 50 to get the total number of spores per bug in 500 µl of water. If no spores were found, the individual was recorded as not being infected. If spores were seen, "low-intensity infection" (< 7.7 x 10^5 spores/insect; < 21 spores/field of view) versus "high-intensity infection" ($\geq$ 7.7 x 10^5 spores/insect; $\geq$ 21 spores/field of view) was recorded.

2.3.4 Physical Signs of Infected BMSB

Brown spots within the abdomen could be seen through the cuticle of BMSB and we evaluated how well these signs indicated *N. maddoxi* infection. BMSB adults included for this analysis were from four sites surveyed in Pennsylvania, one site

surveyed in North Carolina, and one site at the Hudson Valley Research Laboratory (Highland, NY). Sixty-two BMSB with brown spots and seventy randomly selected BMSB that did not have brown spots were included in this evaluation (total = 132 BMSB adults). Microscopy was not used for the observation of the brown spots that were usually found in the center of the abdomen. These brown spots were not part of the cuticular color patterning, which can be variable among individual BMSB.

2.3.5 Statistical Analysis

All statistical analyses were conducted using SAS (SAS Institute 2002-2012). To determine if the prevalence of *N. maddoxi* infection and the prevalence of low- and high-intensity infections were associated with season, Proc Glimmix with a binomial distribution was used. Data from the three collection sites were considered three replicates and a Tukey test was used to test whether the prevalence of infection significantly differed among the seasons. Two-by-two contingency tables were used to compare the prevalence of infection at the three sites used in the phenology study. Comparisons were made between the three sites for each collection during spring, summer, and fall to determine if the overall prevalence differed by site for those collection times. To determine if the prevalence of *N. maddoxi* infection significantly differed between the three sites for these collection times, Fisher's exact test with a two-sided p-value were used with a Bonferroni correction, comparing all of the p-values. Therefore, individual comparisons were tested at the alpha = 0.0167. An additional two-by-two contingency table was used to determine if the number of infected and healthy adults was different than the number of infected and non-infected

nymphs collected during summer collections one and two. Fisher's exact test with a two-sided p-value was used to determine significance.

For the 2017 field survey data, to determine if there was an association between the prevalence *N. maddoxi* infections and BMSB population density, Proc Glimmix with a binomial distribution and logit link were used with site as a random variable. Collections sampled only in the fall from eastern and western sites were used. This analysis did not compare low- or high-intensity infections due to the scarcity of high-intensity infections in these sites. To determine if there were differences in infection intensity according to BMSB sex or presence of native species, Proc Glimmix with a binomial distribution and logit link were used (n=16 sites) for the separate analyses of the overall prevalence of *N. maddoxi* infection, prevalence of low-intensity infections and prevalence of high-intensity infections. Sex and the presence of natives (yes or no) were the predictor variables. Site was set as a random intercept. This analysis only used collections from the fall, as this is when collections occurred at most sites. Proc Glimmix with the site as a random intercept was used to determine whether the prevalence of *N. maddoxi* infection in collected BMSB adults at a site was associated with the number of years BMSB was present in the state where the sample was taken (Hamilton et al. 2018). The number of years BMSB was present in the state where the sample was taken was the predictor variable and the prevalence of *N. maddoxi* infection, the prevalence of low-intensity infections, or the prevalence of high-intensity infections were the response variables.

A two-by-two contingency table was used to analyze the physical signs data. For this test, we compared whether infection prevalence was associated with the

presence of brown spotting. Another two-by-two contingency table was used to determine if the presence of brown spots was associated with the prevalence of high- or low-intensity infections.

2.4 Results

2.4.1 Phenology of N. maddoxi in BMSB Populations in 2018

Overall, the prevalence of *N. maddoxi* in field populations was 21.2% of the 709 individuals sampled from the three sites across the 2018 season. Prevalence ranged from 0.0% to 60.0% depending on location and season (Fig. 2.1A). Peak *N. maddoxi* prevalence (60.0%) and peak infection intensity (43.3% high-intensity infections) were recorded from Hudson Valley in spring (May 2018).

Prevalence of *N. maddoxi* infection by site was significantly associated with season ($F_{3,6} = 27.88$; $P = 0.001$; Fig. 2.1A). For spring collections, when only adults were collected, two of the three sites had the highest infection prevalence for the season, with 52.3-60.0% infection (Fig. 2.1A). Infection levels among adults in fall were lower than spring (6.6-44.0%; Fig. 2.1A). The prevalence of *N. maddoxi* infection was at its lowest during the summer collections, when both adults and nymphs were collected: 9.7 ± 4.1% and 7.3 ± 2.4% for summer collections one and two, respectively (Fig. 2.1A). Low and high infection intensities were both higher in the spring collection (low-intensity infection prevalence: 24.2 ± 12.1% ; high-intensity infection prevalence: 13.2 ± 7.5%) than in summer collection one (low-intensity infection prevalence: 7.9 ± 2.7%; high-intensity infection: 1.8 ± 1.8%) (Fig. 2.1B-C).

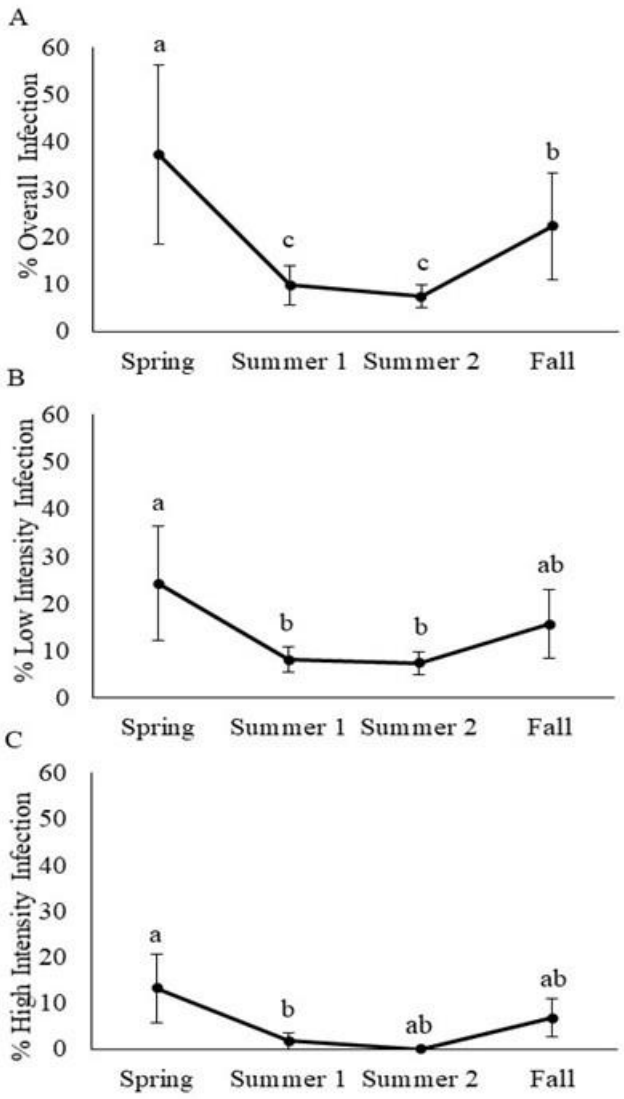

Figure 2.1. Mean (±SE) percentages of the seasonal prevalence of *N. maddoxi* infection at three collection sites in 2018. (A) Mean (±SE) percentages of the overall prevalence of *N. maddoxi* infection at three collection sites for each season. Vertical bars denote standard error estimates associated with each collection from three collection sites. Different letters over data points and bars indicate significant ($P< 0.05$) differences, Tukey test. (B) Mean (±SE) percentages of the prevalence of low-intensity infections at three collection sites for each season. Vertical bars denote standard error estimates associated with each collection from the three collection sites. Different letters over data points and bars indicate significant ($P< 0.05$) differences, Tukey test. (C) Mean (±SE) percentages of the prevalence of high-intensity infection at three collection sites for each season. Vertical bars denote standard error estimates associated with each collection from three collection sites. Different letters over data points and bars indicate significant ($P< 0.05$) differences, Tukey test.

The prevalence of low-intensity infections was significantly different by season ($F_{3,6} = 12.54$; $P = 0.01$; Fig. 2.1B) and not different among sites ($F_{2,6} = 2.60$; $P = 0.1537$). The prevalence of high-intensity infections was also significantly different by season ($F_{3,6} = 6.66$; $P = 0.02$; Fig. 2.1C) and not different among sites ($F_{2,6} = 0.78$; $P = 0.50$). Low-intensity infections were present for all seasons, except in spring for one site, and high-intensity infections were only present at two out of the three sites (Mickley and Hudson Valley) in spring, summer collection one, and fall. Among the 150 infected individuals, overall low-intensity infections (69.3%) were more common than high-intensity infections (30.7%) ($\chi^2 = 22.4$; df = 1; $P < 0.0001$).

In summer collection one, across all sites, the percent of adults that were infected (15.5%, n = 84) was significantly higher than the percent of infected nymphs (5.7%, n = 140) (Fisher's exact test; $P = 0.02$; Fig. 2.2). However, in summer collection two the percent of infected adults (8.2%; n = 85) was not significantly different than the percent of infected nymphs (6.0%; n = 83) (Fisher's exact test; $P = 0.77$) (Fig. 2.2).

The prevalence of *N. maddoxi* infection was also significantly different by site ($F_{2,6} = 8.23$; $P = 0.02$). The one site where the prevalence of *N. maddoxi* infection was lower was Schutt's (Hudson Valley vs Schutt's: t = 4.05; df = 6; $P = 0.01$) (Mickley vs Schutt's: t = 2.84; df = 6; $P = 0.03$) (Hudson Valley vs Mickley: t = 1.48; df = 6; $P = 0.19$).

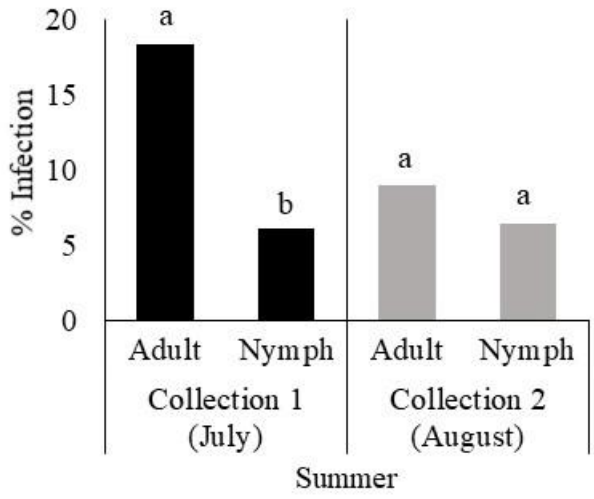

Figure 2.2. Comparisons of the prevalence of *N. maddoxi* infection in BMSB adults and nymphs collected during the summer at three collection sites in 2018. Line between collection 1 and 2 represents separate analyses. Different letters over bars indicate significant (two-sided $P < 0.05$) differences, Fisher's exact test.

2.4.2 Field Prevalence and Intensity of N. maddoxi Infections

The field survey conducted in 2017-2018 revealed that *N. maddoxi* was present in BMSB populations in all states sampled (Fig. 2.3). *Nosema maddoxi* infections were found in 38 of the 47 collections. Six of the eight sites without *N. maddoxi* were in OR, UT, or CA, and the other two sites where *N. maddoxi* was not found were in NY and NC. Two of the nine collections without *N. maddoxi* were sampled in the spring, four out of the nine were sampled in the summer, and three out of the nine were sampled in the fall.

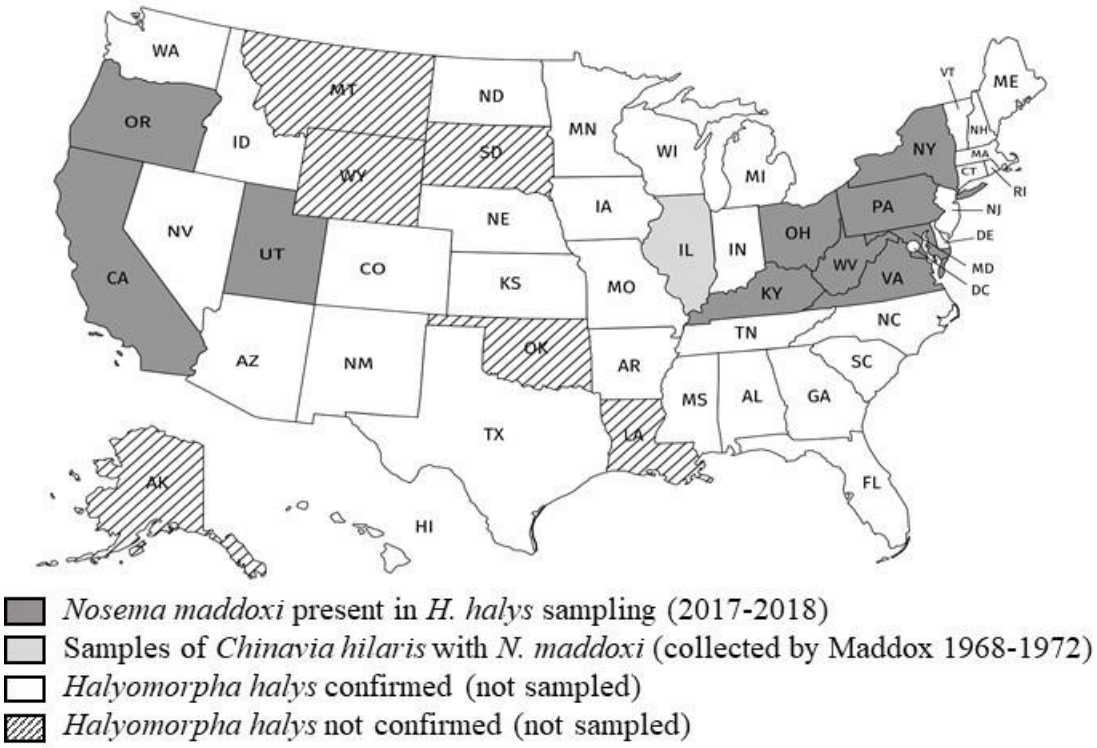

Figure. 2.3. Map of the United States highlighting states in dark grey where *N. maddoxi* was found from the 2017-2018 field samples and from Hajek et al. (2017). Samples with *N. maddoxi* from IL were from green stink bugs collected by Joseph Maddox in 1968-1972 and are highlighted in medium grey. BMSB has been reported from all but 6 US states (Northeastern IPM Center 2018).

Nosema maddoxi infection prevalence averaged 18.9 ± 4.3% by site (range: 0.0-52.0%; n = 2,106 BMSB). Among the 399 infected BMSB, low-intensity infections (62.4%; n = 249) were more common than high-intensity infections (37.6%; n = 150) ($\chi^2 = 24.56$; df = 1; $P < 0.0001$). The overall prevalence of *N. maddoxi* infection was not significantly associated with BMSB population density (Table 2.3). BMSB sex was not associated with overall *N. maddoxi* infection prevalence or with either infection intensity (Table 2.3). The associations between the overall prevalence of *N. maddoxi* infection and the prevalence of either infection intensity with the presence of the three native stink bug species at a site were not significant (Table 2.3)

but there was a numerically positive trend. There was no association between infection prevalence and the number of years BMSB was present in the eastern states where collections were sampled (Table 2.3). However, there was a positive trend for the association of high-intensity infections with the number of years BMSB was present at a collection site, which was marginally insignificant (Table 2.3).

Table 2.3. Analysis of 2017 field survey data. Column labeled "n" represents the number of sites used for each analysis.

Analysis	Infection Prevalence	n	df	*F*	*P*
Population density[a]	Overall	5	1, 3	4.85	0.11
Sex[b]	Overall	16	1, 15	0.59	0.46
	Low intensity	16	1, 15	1.01	0.33
	High intensity	16	1, 15	0.03	0.87
Presence of native stink bugs[b]	Overall	16	1, 15	3.02	0.10
	Low intensity	16	1, 15	1.67	0.22
	High intensity	16	1, 15	2.07	0.12
Years BMSB present[b]	Overall	12	1, 12	1.83	0.20
	Low intensity	12	1, 12	0.44	0.52
	High intensity	12	1, 12	4.68	0.0513

[a] Population density analysis does not include statistical analyses for the prevalence of low- and high-intensity infections due to very low numbers of BMSB with high-intensity infections from the collection sites used for that analysis.
[b] Each analysis includes the statistic for the overall prevalence of *N. maddoxi* infection, the prevalence of low-intensity infection, and the prevalence of high-intensity infection.

2.4.3 Physical Signs of Infected BMSB

Dissections of BMSB with brown spots that were infected revealed that the spots were melanized tissues underneath the abdominal cuticle (Fig. 2.4B-C). The percentage of infected BMSB with brown spots (74.2%) was significantly different from the percentage of infected BMSB without spots (30.0%) ($\chi^2 = 25.69$; $P < 0.0001$)

(Fig. 2.5). Among individuals with spots, there was no difference in the number of individuals with high- versus low-intensity infections ($\chi^2 = 0.29$; $P = 0.59$).

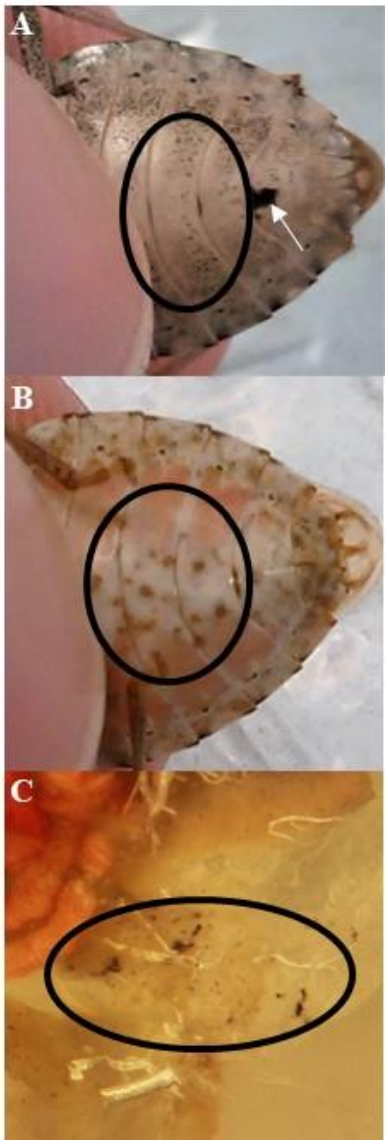

Figure 2.4. Physical signs of *N. maddoxi* infection in BMSB adults. (A) Uninfected BMSB adult without brown spots visible through the cuticle of the abdomen (see circle). The black spot on the abdomen is normal cuticle coloration (see arrow). (B) Infected BMSB adult with brown spots visible through the cuticle of the abdomen (see circle). (C) Dissection of an infected BMSB adult revealing brown melanized tissues (see circle).

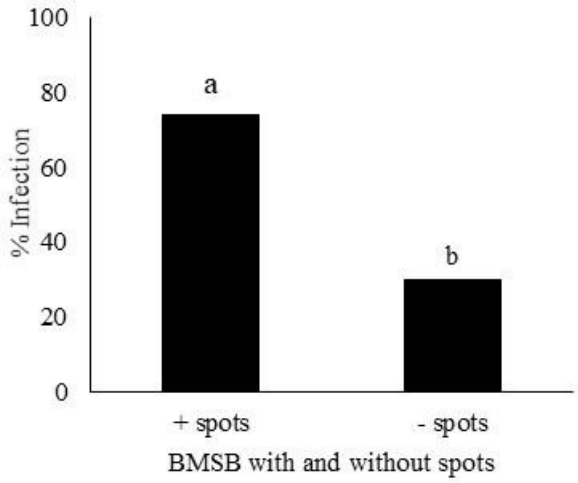

Figure 2.5. Comparison of the percentage of BMSB adults with or without spots that were infected with *N. maddoxi*. Different letters over bars indicate significant ($P < 0.05$) differences, two by two contingency table.

2.5 Discussion

Based on the data collected in 2018, the highest percentage of BMSB that were infected were sampled in spring (50.0-60.0%) and fall (6.6-44.0%) (Fig. 2.1A). This correlates with time periods when BMSB populations are at their peak densities and when they are aggregated (Nielsen et al. 2008). This also indicates that there is seasonality to the prevalence of *N. maddoxi* infection in BMSB populations. One study on the prevalence of *Nosema* spp. (*N. apis* and *N. ceranae*) infections in *A. mellifera* colonies in Europe from 1999-2002 determined that there was a seasonal pattern where the lowest infection levels occurred in the summer, followed by a small peak in the fall, a slow rise during the winter, and a large increase in the spring (Martín-Hernández et al. 2007). However, from 2003-2005, this seasonal pattern changed and lacked seasonality, most likely due to the increase of higher *N. ceranae*

prevalence in Spain in 2005 (Martín-Hernández et al. 2007). Further studies on the phenology of *N. maddoxi* prevalence in BMSB populations would be required to determine if the seasonality of *N. maddoxi* prevalence will change over time.

Data collected in 2018 indicated that infection prevalence in nymphs was lower than in adults in the first summer collection, when there were numerically more nymphs than adults present in the population (Fig. 2.2). However, this was not the case in the second summer collection. This could be due to the different nymphal stages present in the population and the age of the adults when these two collections were made. According to Nielsen and Hamilton (2009), BMSB adults from the overwintering population in Allentown, Pennsylvania, are still present as well as younger nymphs (first instar - third instar) in July (summer collection one). In August (summer collection two) there are fewer younger nymphs and more older nymphs (fourth instar - fifth instar) present, and first-generation adults are present (Nielsen and Hamilton 2009). Adults that were collected in July in this study could be infected adults from the parent generation that overwintered in aggregations before spring dispersal, while adults collected in August were from the new generation and may have been starting to be infected by *N. maddoxi*. One study on *Nosema pyrausta* Paillot prevalence in *Ostrinia nubilalis* (Hübner) determined that the disease development in first-generation *O. nubilalis* populations had slower rates of infection during larval development (Andreadis 1984). This could explain why there were lower levels of infection in BMSB nymphs. In addition, early larval or nymphal life stages are often more susceptible to microsporidian infection than later life stages (Hoch and Solter 2018). Our study on the effects *N. maddoxi* on BMSB nymphs observed that

56% of treated young BMSB nymphs infected with *N. maddoxi* died before they reached the fourth instar (see Chapter 3). Thus, nymphal infection seemed to decrease (compared to adult infection) in August, perhaps due to poor survival.

BMSB adults that are preparing to overwinter aggregate into large groups in fall, and this could increase the possibility for horizontal transmission of *N. maddoxi*. Overwintering adults also remain in groups at overwintering sites from late September to March through June when they disperse depending on location, dispersing later in areas in the northern US and earlier in areas in the southern US (Nielsen et al. 2016). Adults that were infected before overwintering could contaminate overwintering sites with *N. maddoxi* spores, resulting in infection of healthy overwintering BMSB adults. Infections in overwintering BMSB adults could also progress from low into high-intensity infections while the BMSB adults overwinter either from progression of infections or reinfection. Some microsporidian species, such as *N. pyrausta* and *Nosema fumiferanae* (Thomson) Percy have been known to persist in host populations by overwintering in infected hibernating hosts (Andreadis 1986; van Frankenhuyzen et al. 2007). This supports the high infection levels found from our phenology study, where two out of the three sites had infection levels between 52.3-60.0% during spring, when only overwintering adults were present (Fig. 2.1A). Therefore, the best time to collect BMSB to detect *N. maddoxi* infection would be in the fall during aggregation and in the spring when adults begin to emerge from overwintering sites. In addition, collecting adults would increase the possibility of finding *N. maddoxi* in a BMSB population instead of collecting nymphs.

Nosema maddoxi was found in every state where collections were made. Since *N. maddoxi* was found in states on both the East and West Coasts and is a native pathogen of green stink bugs, brown stink bugs, and dusky stink bugs, we hypothesize that this microsporidian species is distributed in BMSB populations throughout the United States, wherever BMSB is established (Fig. 2.3).

In general, microsporidia are considered to be host density-dependent (Hoch and Solter 2018) . The prevalence of *N. pyrausta*, a pathogen of the European corn borer (*O. nubilalis*), was found to be dependent on the density of its host and therefore this microsporidian played an important role as a natural biological control agent (Andreadis 1984). A study on jack pine budworm (*Choristoneura pinus* Freeman) examined the prevalence of *Nosema* sp. in the field and determined that the prevalence of *Nosem*a sp. increased as time progressed and the host populations with high *Nosema* sp. infection prevalence did not expand to other areas (van Frankenhuyzen et al. 2011). In this study, the prevalence of *N. maddoxi* infection was variable among sites and was found across a range of BMSB population densities. *Nosema maddoxi* is known to infect BMSB by horizontal transmission (Hajek et al. 2017; Chapter 3). To become infected via horizontal transmission, healthy bugs must ingest spores (Becnel and Andreadis 2014). Horizontal transmission of microsporidia can occur by feeding on infected eggs, contaminated food, and cadavers (Solter et al. 2012a). In the lab, BMSB have been observed to feed on eggs, nymphs, freshly eclosed adults, and cadavers (Medal et al. 2012). We hypothesized that at sites with higher BMSB population densities, BMSB individuals would be more likely to come into contact with each other while feeding, searching for mates, during aggregation in the fall, and

during overwintering, and as a result the prevalence of *N. maddoxi* infection at those sites would be higher compared to sites with lower BMSB population densities. However, we did not observe this in our study, perhaps because the sample size to determine if BMSB population density was associated with *N. maddoxi* infection prevalence was small (n = 5 sites). Increasing the sample size could provide more information on these associations.

Microsporidian infections have been observed to cause external and internal effects in their hosts (Becnel and Andreadis 2014). One effect is the formation of melanized areas in tissues as a result of the host's immune response (Becnel and Andreadis 2014, Hoch and Solter 2018). This could explain why *N. maddoxi* spores were found in most of the BMSB with brown spots beneath the abdominal cuticle. However, microsporidian infections do not always provoke the host immune response (Hoch and Solter 2018) which agrees with our finding where not all infected BMSB had brown spots. The formation of melanized tissue also takes time (Tokarev et al. 2006). Infected crickets (*Gryllus bimaculatus* De Geer) did not present melanized tissue until 60-90 days after being infected by *Paranosema grylli* (Sokolova, Selezniov, Dolgikh, Issi) Sokolova et al., and infected locusts (*Locusta migratoria* (L.)) did not present melanized tissue until 14-20 days after being infected by *Antonospora locustae* (Canning) Slamovits, Williams & Keeling (Tokarev et al. 2006). In our study, BMSB were collected from the field and we could not determine when they had initially been infected by *N. maddoxi*. We hypothesize that BMSB that were infected and had brown spots were probably infected for a longer time span than infected BMSB that did not have brown spots. The presence of brown spots in BMSB

collected in the field could be considered an approximate indicator of *N. maddoxi* infection, but microscopy or molecular methods should be used for confirmation since brown spots occurred in some individuals in which we did not find *N. maddoxi* spores and not all infected BMSB had brown spots. Causes of the brown spots found in the BMSB that did not contain spores were not determined in our study.

Nosema maddoxi infections were not detected in all collections of BMSB, but this does not mean that this pathogen was not present at those sites. In 2017, one-four collections were sampled from each site, and 50 BMSB from most of the collections were used for diagnoses. Taking multiple collections from a site and increasing the number of BMSB used for microscopy could improve the possibility of finding *N. maddoxi* infections if prevalence was low. In addition, microscopy may not be the best method for detecting microsporidian infection. Sokolova et al. (2004) reported results comparing four methods for detection of microsporidian infections in fire ants (*Solenopsis invicta* Buren). Methods included examination of smears under a microscope using phase contrast, Giemsa and Chromotrope-based trichrome staining, and PCR. It was determined that the Chromotrope-based trichrome staining was the best method for diagnosing microsporidian infections, followed by PCR and Giemsa staining, and the least sensitive was checking smears under phase contrast microscopy. Detection of microsporidian infections by checking smears using phase contrast was only effective when the concentration of spores was more than 10^6 spores/ml. Also, pre-spore developmental stages could be overlooked and misidentified when using smears with phase contrast. However, a smear processed by Chromotrope-based trichrome staining, could detect infections with spore concentrations that ranged

between 5.0 x 10^2-10^3 spores/ml and pre-spore developmental stages. With Giemsa staining pre-spore development was detectable, but could be mistaken as debris or cell organelles, and the intense staining of the background led to underestimating the number of microsporidia in the smear. Therefore, depending on the experience of the investigator, Giemsa staining was effective if the spore concentration ranged from 10^4-10^6 spores/ml. PCR was also very effective in detecting microsporidian infections that had a spore concentration of 10^3 spores/ml (Sokolova et al. 2004). Based on these findings, using Chromotrope-based trichrome staining or PCR, could increase the number of positive *N. maddoxi* infections. However, in this study 2,815 bugs were evaluated, and these methods are more time consuming and expensive than checking bug smears with a microscope using phase contrast. In addition, to examine and diagnose all of the BMSB samples, they were frozen until they could be diagnosed, and the majority of specimens were sent dead. The use of live infected insects is the best way to examine microsporidia, but most terrestrial host microsporidia are known to have some degree of tolerance to desiccation and freezing (Solter et al. 2012b). However, due to the number of samples sent from different sites that were handled by several people, the viability of *N. maddoxi* in those samples was uncertain. So, using these more sensitive methods would not have been ideal for this study.

In summary, the native microsporidian *N. maddoxi* is widely distributed in BMSB populations in the US. Some microsporidian species are influential in providing control of host populations (Andreadis 1984, van Frankenhuyzen et al. 2011, Becnel and Andreadis 2014, Bjørnson and Oi 2014). The population level impact by *N. maddoxi* on BMSB population density has not been determined, but the

prevalence of *N. maddoxi* was found to be seasonal and was highest in the spring when BMSB are preparing to disperse from overwintering sites. The seasonality of *N. maddoxi* in BMSB populations could impact BMSB population dynamics and could provide information on the transmission of this microsporidian species.

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CHAPTER 3

Impact of *Nosema maddoxi* on the Survival, Development, and Female Fecundity of *Halyomorpha halys*[2]

3.1 Abstract

Nosema maddoxi, a microsporidian species native to the United States, was recently found to infect the invasive brown marmorated stink bug (*Halyomorpha halys*). Some microsporidian species have been known to negatively impact their hosts by shortening the lifespan of adults, causing nymphal mortality, decreasing female fecundity and viability of eggs, prolonging nymphal development, and stunting growth. This study was conducted to determine what effects *N. maddoxi* has on *H. halys*, specifically on *H. halys* adult females and nymphs, using inoculum of two spore concentrations. Treated *H. halys* females in the low spore concentration bioassay had low- (45.8%) or high-intensity (54.2%) infections and there was no significant difference in survival between treated females and controls. The majority of treated *H. halys* females in the high spore concentration bioassay had high-intensity infections (82.6%) and treated females died significantly faster than controls. Treated nymphs died significantly faster than control nymphs. Of the treated nymphs, 56% died before

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molting into the fourth instar and only 26% eclosed to adults. *Nosema maddoxi* infections negatively impacted female fecundity in both adult spore concentration bioassays. Treated females in the low spore concentration bioassay produced significantly fewer eggs per egg mass (23.1 ± 0.6 eggs per egg mass) than controls (25.1 ± 0.6 eggs per egg mass), but there were no significant differences in the total number of eggs laid, and in the total number of eggs masses laid. Treated females in the high spore concentration bioassay laid significantly fewer total eggs (62.5 ± 7.8 eggs) than controls (149.8 ± 10.1 eggs), significantly fewer egg masses (2.9 ± 0.3 egg masses) than controls (5.5 ± 0.3 egg masses), and significantly fewer eggs per egg mass (22.0 ± 1.2 eggs per egg mass) than controls (26.3 ± 0.6 eggs per egg mass). Egg viability was significantly lower in treated females compared with the controls for both adult spore concentration bioassays. Nymphal development rate and size were not impacted by *N. maddoxi* infection. These results indicate that *N. maddoxi* infection does negatively impact the lifespan of adult females, female fecundity, egg viability, and nymphal survival with the potential to impact *H. halys* populations.

3.2 Introduction

Nosema maddoxi, a microsporidium, is an entomopathogen of the invasive brown marmorated stink bug (*Halyomorpha halys*) (Hajek et al., 2017). In 1968, Joseph Maddox discovered this microsporidian parasitizing native green stink bugs (*Chinavia hilaris*) in Illinois (Maddox, 1979) before *H. halys* had been discovered in North America (Hajek et al., 2017). A field study in 2017-2018 determined that *N. maddoxi* had a wide distribution in *H. halys* populations as it was found in every state

where *H. halys* samples were collected (CA, KY, MD, NC, NY, OH, OR, PA, UT, VA, and WV), with infection prevalence varying by season and ranging from 0-60% (see Chapter 2).

Halyomorpha halys was introduced from Asia and has currently been found in 44 states and four Canadian provinces (Northeastern IPM Center, 2018). Management of *H. halys* populations has mostly been dependent on the use of insecticides (Abram et al., 2017). Some integrated pest management (IPM) strategies that include the use of insecticides and biological control agents have been developed to control *H. halys* populations in agriculture. One biological control agent that has been successful in its native range and has potential in the United States is *Trissolcus japonicus*, the samurai wasp (Abram et al., 2017). To study potential biological control agents and other control methods, lab colonies of *H. halys* were produced, but some colonies collapsed due to high levels of infection caused by the microsporidian *N. maddoxi* (Hajek et al., 2017).

Microsporidian infections are usually chronic; effects may include a shortened lifespan, a decrease in fecundity, prolonged development and stunted growth (Becnel and Andreadis, 2014; Hoch and Solter, 2018). In field studies, some microsporidian species have been confirmed to have an impact on insect populations (Andreadis, 1984; van Frankenhuyzen et al., 2011; Bjørnson and Oi, 2014; Hopper and Mills, 2016). For example, *Paranosema locustae*, a microsporidian species that parasitizes several grasshopper species, has been developed as a biopesticide and is commercially available in the United States (Bjørnson and Oi, 2014).

This study focused on the effects *N. maddoxi* has on *H. halys* adult females and nymphs. Objectives included testing adult female and nymphal survival, female fecundity, egg viability, and nymphal development. Based on other studies, we predicted that infected females would have shorter lifespans, and that fecundity and egg viability would be significantly lower than in non-infected females (Solter et al., 2012a; Hopper et al., 2016). We also expected that *H. halys* nymphs would be susceptible to *N. maddoxi*, have stunted growth, and prolonged development, which has been reported in other insect species infected by microsporidia (Solter et al., 2012a; Gupta et al., 2016; Hopper et al., 2016; Hoch and Solter, 2018).

3.3 Materials and Methods

3.3.1 Halyomorpha halys Source and Maintenance

Halyomorpha halys used for studies were obtained from lab colonies maintained at USDA-ARS IIBBL, New Jersey Department of Agriculture, Beneficial Insect Laboratory, and North Carolina State University, Mountain Horticultural Crops Research & Extension Center. When *H. halys* were received from these colonies, a subsample (~5-8 *H. halys*) was checked to confirm that the *H. halys* received were not infected by microsporidia. Thereafter, irregular checks of our *H. halys* colonies were conducted before *H. halys* were used in bioassays, especially if unexpected mortality occurred.

Rearing methods for *H. halys* colonies used throughout this study were similar to the methods described by Herlihy et al. (2014). Organic carrots were an additional food source, and organic unsalted sunflower seeds and organic peanuts were used

instead of the seed paper used by Herlihy et al. (2014). Food for the *H. halys* colony was replaced every other day. Our *H. halys* colonies were held in a growth chamber at 25ºC, with a 16-hour photoperiod (16:8 h L:D), and with 50-55% relative humidity.

Halyomorpha halys used in two replicates of the adult low spore concentration bioassay and second instar nymphs were reared from *H. halys* eggs. *Halyomorpha halys* used in the third replicate of the adult low spore concentration bioassay and all replicates of the adult high spore concentration bioassay were sent as fifth instars and were reared until they eclosed into adults for bioassays. Colonies of non-infected *H. halys* males were maintained throughout the study.

3.3.2 Spore Suspension and Spore Concentration Preparation

Field-caught *H. halys* adults collected in Biglerville, PA, that were infected by *N. maddoxi* were used to prepare *N. maddoxi* spore suspensions. The methods used to obtain microsporidian spore suspensions and for long-term storage were based on methods described by Solter et al. (2012b). Hemocytometer counts were used to determine the spore concentration of the spore suspensions used for bioassays. Spores were quantified using a microscope at 400x with phase contrast. For the adult low spore concentration bioassay, two replicates had a spore concentration of 1.4×10^7 spores/ml and one replicate had a spore concentration of 1.6×10^7 spores/ml. Individual females in the two replicates that had a spore concentration of 1.4×10^7 spores/ml were fed a spore suspension of 350,000 spores and individual females in the replicate that had a spore concentration of 1.6×10^7 spores/ml were fed a spore suspension of 400,000 spores. For the high spore concentration bioassay, the spore concentration was 3.5×10^7 spores/ml and individual females were fed a spore

suspension of 875,000 spores. The spore concentration used for the nymphal bioassay was 1.4 x 10^7 spores/ml and a spore suspension of 28,000 spores was fed to each nymph.

3.3.3 Adult Female Bioassays

Adult female *H. halys* (1-6 days after eclosion) were randomly collected from the colony, placed into individual 2-oz SOLO soufflé plastic cups with a layer of parafilm on the bottom, and half of an organic peanut, and were covered by a lid. Containers were kept in a growth chamber at 25ºC with a 16-hour photoperiod (16:8 h L:D) and 50-55% relative humidity for 24 hours. After 24 hours, treatment females were separated from controls and both groups were then given the treatments.

For treatment, a 25-µl droplet of the spore suspension was pipetted into each plastic cup for the adult low and high spore concentration bioassays. Controls were given a 25-µL water droplet for the adult low and high spore concentration bioassays. Each female was observed every 15 minutes for 3 hours. Only females who finished imbibing the entire droplet continued in the study and were placed into individual 16-oz plastic deli cup containers. Three replicates were conducted for the low spore concentration bioassay (treatment: n = 48 and control: n = 33). Four replicate were conducted for the high spore concentration bioassay (treatment: n = 35 and control: n = 43). The pronotum widths of the females in the high spore concentration bioassay were measured after they were inoculated. Each container was lined with circular filter paper (9.0 cm diam.) on the bottom, and half of an organic green bean, half of an organic carrot, organic raw sunflower seeds, and a 1.5-ml Eppendorf tube of tap water were placed on top of the filter paper. Containers were labeled with the identification

number of the female that was inoculated and were placed back into the growth chambers. Food and water were replaced every other day.

When the oldest females reached 8 days after eclosion, a healthy male was added to each container. Containers were checked daily for eggs, which were counted and collected. All eggs were separately maintained by female and day in individual 100 x 15-mm petri dishes with moistened 9.0-cm filter paper. Each petri dish was wrapped with parafilm. Eggs were placed in the same growth chamber where the treatments or controls were maintained for 5-10 days, depending on whether the eggs were from a treated or control female. Eggs were checked for hatch daily. For egg viability, the numbers of first instars and unhatched eggs were recorded.

Containers were checked daily for mortality. If males housed with females were found dead but the female was alive, the male was discarded and replaced with a healthy male from the colony. If the female was found dead, the male was discarded and the female was collected and stored at -20ºC until diagnosis by microscopy. Females in the adult spore concentration bioassays that were still alive 42-44 days after inoculation were stored at -20ºC until diagnosis by microscopy.

3.3.4 Nymphal Development Bioassay

Second instars, 2-3 days after molting, were isolated similarly to the adult female bioassays for 16 hours and provided half a peanut. Nymphs were given a 2-µL droplet of the spore suspension, or a 2-µL water droplet and were observed for 1 hour. Only nymphs that drank all of the droplet continued in the study. Three replicates were conducted for the nymphal bioassay (treatment n = 34; control n = 40). Nymphs were monitored daily and the day of death was recorded. The days a nymph molted the

pronotum width (mm) was measured. The bioassay was ended at 21 days after eclosion into adults.

3.3.5 Microscopy

To evaluate the presence of *N. maddoxi* infection and intensity of infection, treated and control adult females were suspended in 500-µl of water in a 1.5-ml Eppendorf tube, and treated and control nymphs were suspended in 200-µl of water in a 1.5-ml Eppendorf tube and both were crushed using a micropestle 8-10 times. Bug suspensions were viewed at 400x with phase contrast. Ten microliters of the suspension was placed on a microscope slide under an 18 mm x 18 mm coverslip. Five fields of view (total area viewed = 0.98 mm^2) were examined per individual to determine whether microsporidia spores were present in the sample and, if spores were present, to determine the infection intensity. For adults, three categories were created: no spores, low intensity infection ($< 7.7 \times 10^5$ spores per insect; < 21 spores/field of view) and high intensity infection ($\geq 7.7 \times 10^5$ spores per insect; $\geq$ 21 spores/field of view). For nymphs, infection presence or absence was used to determine whether treated nymphs were infected, and infection intensity was not determined.

3.3.6 Data Analysis

All data were analyzed using SAS (SAS Institute, version 9.4, 2002-2012). All statistical analyses for the adult low spore concentration bioassay and for the high spore concentration bioassay compared differences among the controls and treated females. Statistical analyses for the nymphal bioassay compared differences among the controls and treated nymphs. Only adult females that lived $\geq$ 8 days after

inoculation and nymphs that lived for ≥ 5 days after inoculation were included in the analyses. Control nymphs that remained as second instars for ≥ 18 days after inoculation were excluded due to irregularity in the amount of time it took to molt into the next instar.

For all survival analyses, PROC LIFETEST was used to calculate the LT_{50} and confidence intervals and PROC PHREG was used to compare the treatment survival with control survival.

For the adult low spore concentration bioassay, PROC MIXED was used to analyze the average egg mass size, total number of egg masses laid, and the average number of eggs produced per day, with replicates as random effects. The data for the total number of eggs laid was log-transformed. To determine the viability of the eggs that were produced, PROC GLIMMIX with a binomial distribution was used. To analyze egg production throughout a female's lifetime, PROC MIXED was used to determine whether there was an association between the treatment and the day when eggs were laid; replicates, the interaction of replicates and female identification, and the interaction of date, female identification, and replicates were random effects. PROC GLIMMIX, with a binomial distribution and logit link was used to determine whether there was an association between the treatment and the day when eggs hatched throughout a female's lifetime; replicates, the interaction of replicates and female identification, and the interaction of date, female identification, and replicates were random effects. Only females that produced eggs were included in all analyses testing fecundity, and only females that produced viable eggs were included in the viability analyses.

The statistical analyses of the fecundity and egg viability of adult females in the high spore concentration bioassay focused on the same variables as the adult low spore concentration bioassay. However, analyses of egg production throughout a female's lifetime and for the viability of eggs produced per egg mass laid throughout their lifetime were not included for this bioassay. All fecundity analyses used the same statistical programs as the adult low spore concentration bioassay, except the data for total eggs laid was not log-transformed, and the data for average egg mass size was analyzed by a nonparametric Wilcoxon Two-Sample test.

To determine whether pronotum width of females in the high spore concentration bioassay reflected how long females lived depending on the treatment, PROC MIXED was used, with replicates and the interactions between days alive and treatment as random intercepts.

For the nymphal bioassay there were two statistical analyses. To determine whether the pronotum width of each instar, after the nymphs had molted, was associated with the treatment, PROC MIXED was used, with replicates and the interaction of replicates and individual nymphs as random intercepts, and pronotum width for each instar as the dependent variable. The second analysis was conducted to determine whether nymphs that were infected had prolonged development. PROC MIXED was used, with replicates as the random intercept and the number of days per instar as the dependent variable.

3.4 Results

3.4.1 Survival Analyses

In the adult low spore concentration bioassay, treated females did not live significantly longer than controls (χ^2 =2.0854; df = 1; P = 0.1487; Fig. 3.1) and replicates were significantly different (χ^2 = 8.4966; df = 2; P = 0.0143). Controls in the low spore concentration bioassay lived for an average of 35.5 ± 1.8 days with no median lethal time, 25% mortality at 30 days (95% confidence interval: 14 - 39 days), and 42.4% died by the end of the study. Treated females lived for an average of 31.0 ± 1.7 days, with an LT_{50} = 36 days (95% confidence interval: 25 – 42 days); 64.6% died by the end of the study (Fig. 3.1). Equal numbers of treated females had low-intensity infections (45.8%) and high-intensity infections (54.2%) (χ^2 = 0.3333; df = 1; P = 0.5637). The percentage of treated females with low-intensity infections was highest (100%) when treated females died 0-10 days after inoculation, and the percentage of treated females with high-intensity infections was highest (100%) when treated females died 41-44 days after inoculation (Fig. 3.2).

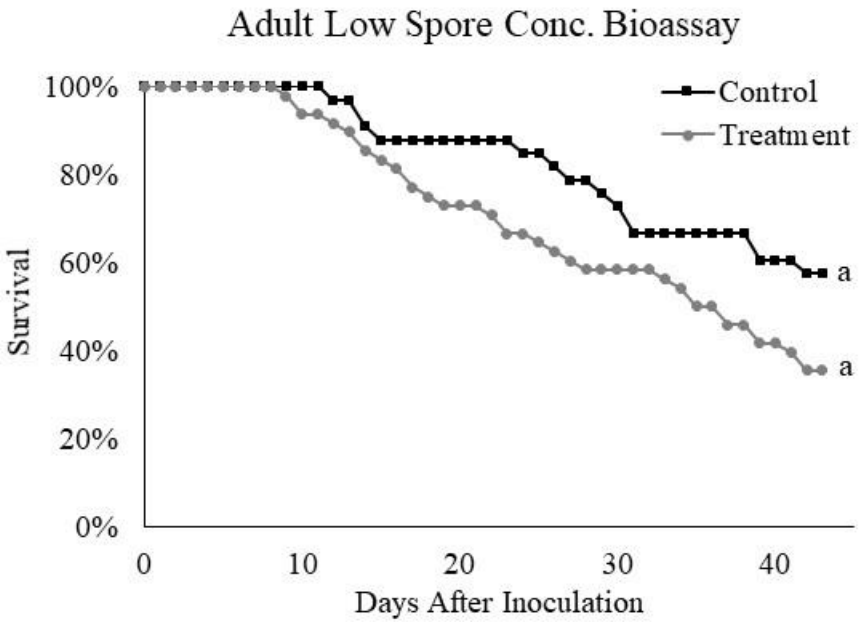

Figure 3.1. Percent survival of female *H. halys* in the adult low spore concentration bioassay. Same letters next to survival curves indicate non-significant (PROC PHREG, $P > 0.05$) differences.

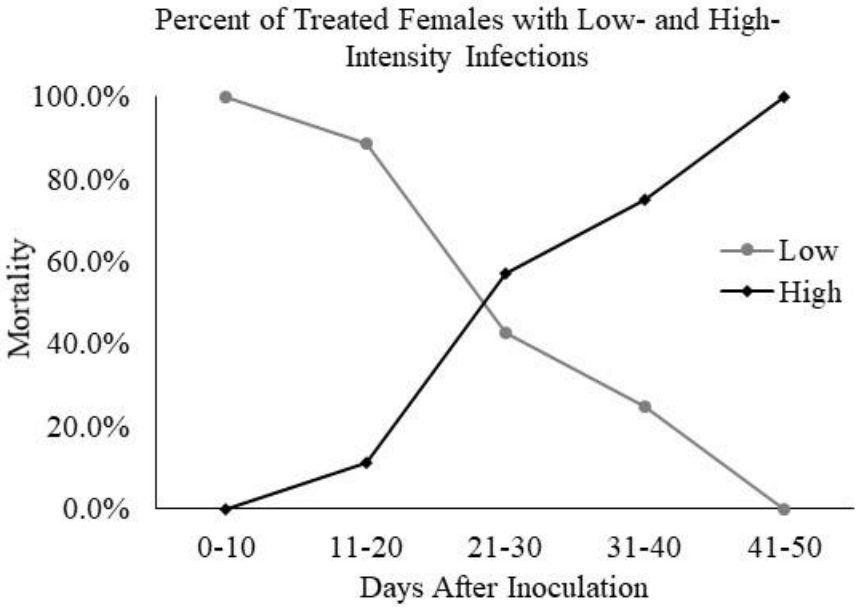

Figure 3.2. Percent mortality of treated *H. halys* females with low- and high-intensity infections in the adult low spore concentration bioassay. Days after inoculation categories represent the percent of treated adult females that died with a low- or high-intensity infection within those time frames.

Treated females in the high spore concentration bioassay died significantly faster than the controls ($\chi^2 = 18.6290$; df = 1; $P < 0.0001$; Fig. 3.3). Controls lived for an average of 33.8 ± 1.1 days, with 74.4% of control females surviving through the bioassay, and 25% of control females died by 37 days (95% confidence interval: 21 - NA days). Treated females lived for an average of 29.3 ± 1.9 days with an $LT_{50} = 32$ days (95% confidence interval: 24 - 36 days); 80.0% died by the end of the study. Most treated females, when diagnosed, had high-intensity infections (82.9%) and only 17.1% of treated females had low-intensity infections.

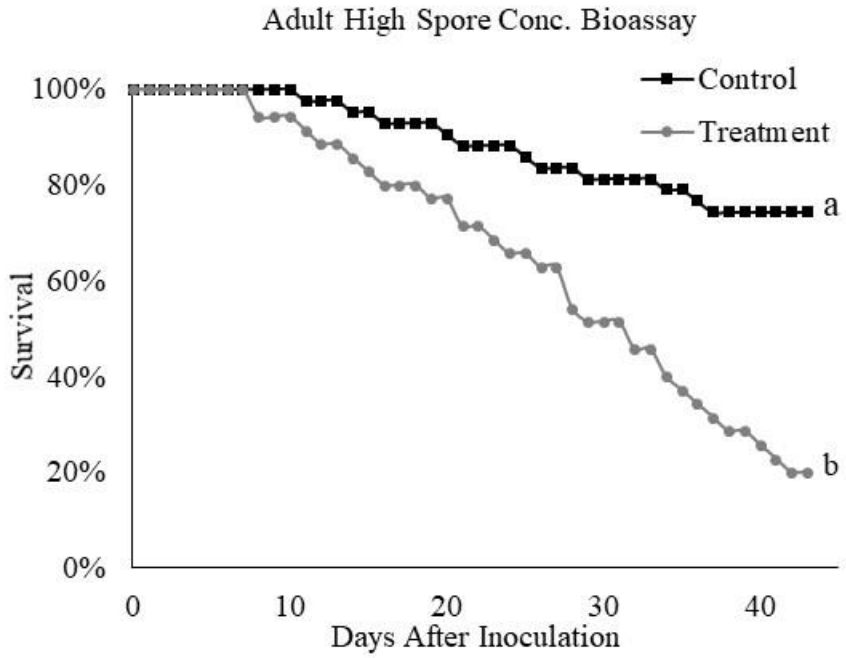

Figure 3.3. Percent survival of female *H. halys* in the adult high spore concentration bioassay. Different letters next to survival curves denote significant (PROC PHREG, $P < 0.05$) differences.

Treated nymphs died significantly faster than control nymphs (χ^2 =10.1469; df = 1; P = 0.0014; Fig. 3.4). Control nymphs lived for an average of 55.0 ± 2.9 days, with an LT_{50} = 66 days (95% confidence interval: 56 - 67 days) and treated nymphs lived for an average of 31.5 ± 4.3 days with an LT_{50} = 18 days (95% confidence interval: 16 - 39 days). For the controls, 75% died as adults, with 3% and 5% of nymphs dying as second and third instars, respectively (Fig. 3.5). For treated nymphs, 26% died as adults, with 32 % and 24 % of nymphs dying as second and third instars, respectively (Fig. 3.5).

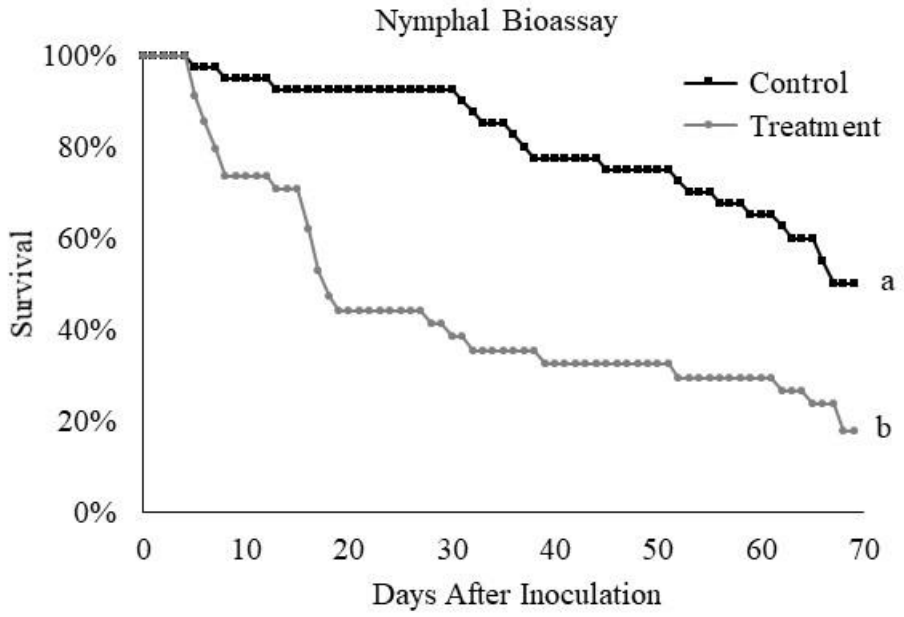

Figure 3.4. Percent survival of *H. halys* nymphs in the nymphal bioassay. Different letters next to survival curves indicate significant (PROC PHREG, $P < 0.05$) differences.

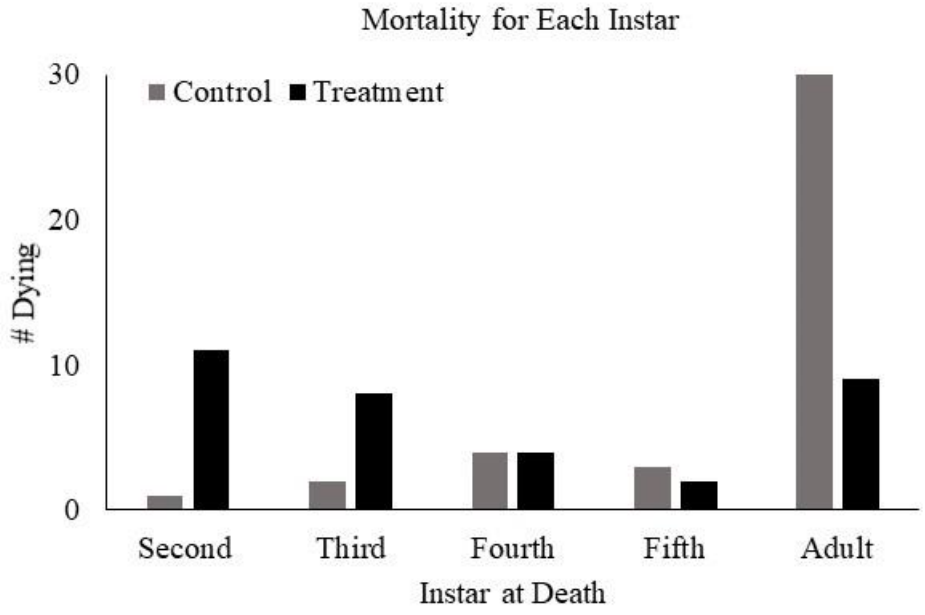

Figure 3.5. The total number of *H. halys* nymphs of each instar that died in control and treatment during the nymphal bioassay.

3.4.2 Low Spore Concentration Bioassay: Female Fecundity and Egg Viability

Treated females receiving the low spore concentration did not lay significantly more eggs ($F_{1,73} = 3.61$; $P = 0.0613$; Table 3.1) and did not lay significantly more egg masses than control females ($F_{1,73} = 1.61$; $P = 0.2080$; Table 3.1). Average egg mass size was significantly smaller for treated females than for controls ($F_{1,73} = 5.38$; $P = 0.0232$; Table 3.1). Treated females did not produce significantly more eggs per day than control females ($F_{1,73} = 1.42$; $P = 0.2377$; Table 3.1). Egg viability was significantly lower for treated females than for the controls ($F_{1,68} = 5.72$; $P = 0.0196$; Table 3.2).

Table 3.1. Mean values (±SE) female fecundity in the adult low spore concentration bioassay.[a]

		Total Eggs Laid[b]	Total Egg Masses[b]	Egg Mass Size[b]	Egg Prod./day[b]
	n	Mean (±SE)			
Control	31	191.3 (15.0) a	7.6 (0.5) a	25.1 (0.6) a	6.4 (0.3) a
Treatment	46	145.4 (12.2) a	6.2 (0.5) a	23.1 (0.6) b	5.8 (0.3) a

[a] Untransformed means and standard errors (±SE) in each column.
[b] Means within columns followed by different letters denote significant differences (Linear mixed model, $P \leq 0.05$).

Table 3.2. Mean (±SE) of overall egg viability (%) of the total number of eggs laid in the adult low spore concentration bioassay.

		Overall Egg Viability (%)[a]
	n	Mean (±SE)
Control	30	84.0 (2.1) a
Treatment	42	83.8 (1.4) b

[a] Means within columns followed by different letters denote significant differences (Generalized linear mixed model, $P \leq 0.05$).

3.4.3 Low Spore Concentration Bioassay: Egg Production and Egg Viability Over Time

Egg production per day over time significantly decreased for treated females as they aged ($F_{1,67.4} = 5.51$; $P = 0.0219$; Fig. 3.6) and the day eggs were produced was significantly different ($F_{1,501} = 21.87$; $P < 0.0001$). Percent viability of eggs produced over time decreased as the females aged and was not significantly different between controls and treated females ($F_{1,408} = 1.13$; $P = 0.2892$; Fig. 3.7), but egg viability varied significantly by day ($F_{1,408} = 131.48$; $P < 0.0001$).

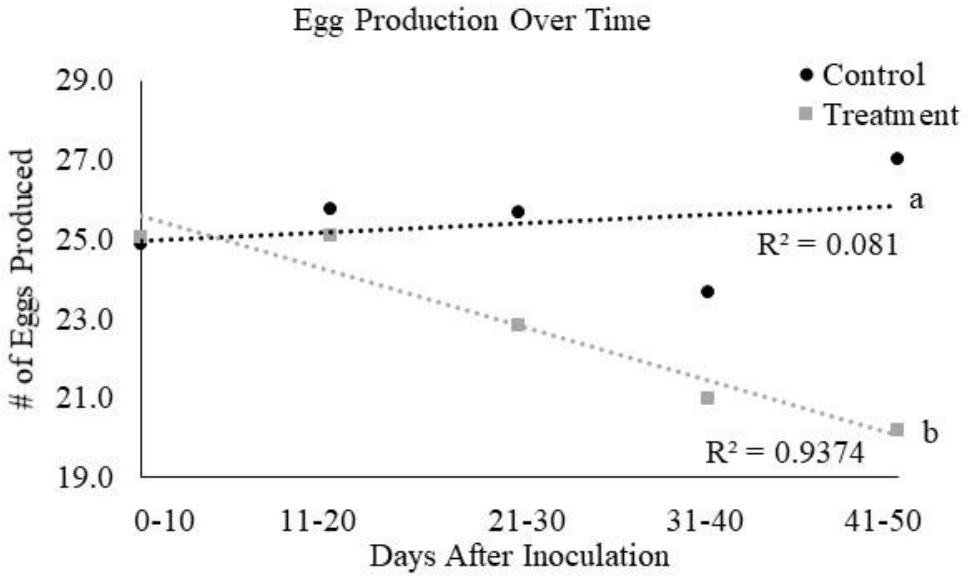

Figure 3.6. Egg production over the lifespan of control and treated *H. halys* females in the adult low spore concentration bioassay. Days after inoculation ranges on the x-axis represent the average number of eggs produced per female from 0-10, 11-20, 21-30, 31-40, and 41-50 days after inoculation. Different letters next to treandlines indicate significant (Linear mixed model, $P \leq 0.05$) differences.

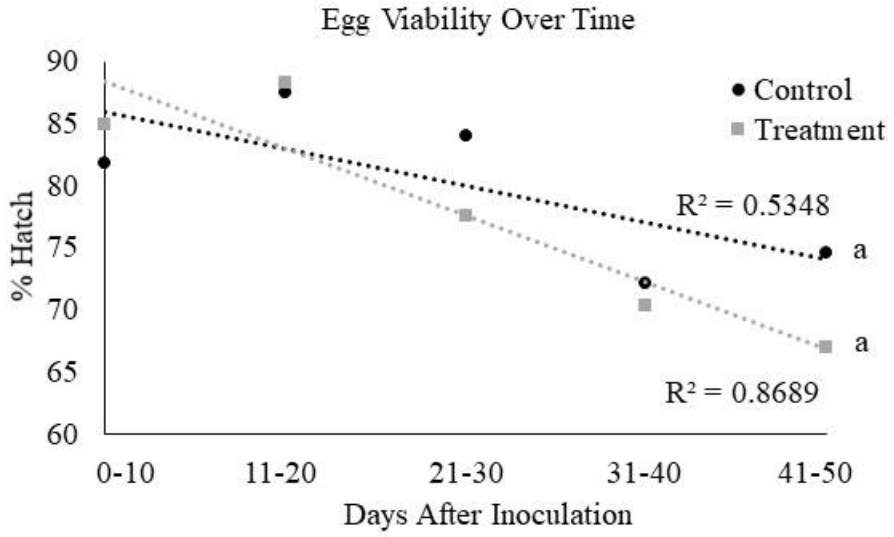

Figure 3.7. Percent viability of eggs produced over the lifespan of control and treated *H. halys* females in the adult low spore concentration bioassay. Days after inoculation ranges on the x-axis represent average % of viable eggs per female from 0-10, 11-20, 21-30, 31-40, and 41-50 days after inoculation. Same letters next to treandlines indicate non-significant (Generalized linear mixed model, $P \geq 0.05$) differences.

3.4.4 High Spore Concentration Bioassay: Female Fecundity and Egg Viability

Treated females laid significantly fewer eggs than controls ($F_{1,64}$= 37.5; $P < 0.0001$; Table 3.3). Treated females also laid significantly fewer egg masses in their lifetime than control females ($F_{1,64}$= 29.32; $P < 0.0001$; Table 3) and produced significantly fewer eggs per egg mass than control females (z = -3.8277; $P = 0.0001$; Table 3.3). Egg production per day was not significantly different between treatment and control females ($F_{1,64} = 0.61$; $P = 0.4365$; Table 3.3). Viability of the eggs produced by treated females was significantly lower than that of the eggs produced by control females ($F_{1,63} = 97.89$; $P < 0.0001$; Table 3.4).

Table 3.3. Mean (±SE) female fecundity in the adult high spore concentration bioassay.[a]

		Total Eggs Laid[b]	Total Egg Masses[b]	Egg Mass Size[c]	Egg Prod./day[b]
	n	Mean (±SE)			
Control	41	149.8(10.1) a	5.5(0.3) a	26.3(0.6) a	5.5(0.3)a
Treatment	28	62.5(7.8) b	2.9(0.3) b	22.0(1.2) b	4.9(0.9)a

[a] Untransformed means and standard errors (±SE) in each column.
[b] Means within columns followed by different letters denote significant differences (Linear mixed model, $P \leq 0.05$).
[c] Means within columns followed by different letters denote significant differences (Wilcoxon two-sample test, $P \leq 0.05$).

Table 3.4. Mean (±SE) of overall egg viability (%) of the total number of eggs laid in the adult high spore concentration bioassay.

		Overall Egg Viability (%)[a]
	n	Mean(±SE)
Control	40	84.0 (2.0) a
Treatment	28	71.1 (4.3) b

[a] Means within columns followed by different letters denote significant differences (Generalized linear mixed model, $P \leq 0.05$).

3.4.5 High Spore Concentration Bioassay: Female Size

The association between female pronotum size and the number of days females were alive did not differ between treatment and controls ($F_{1,71} = 1.31$; $P = 0.2557$).

3.4.6 Nymphal Development

Nymphal pronotum size for each instar was not significantly different between treatment and controls ($F_{4,148} = 0.86$; $P = 0.4882$). There were no significant differences between the number of days for treated and control nymphs to molt from the second to third instar ($F_{1,35} = 0.40$; $P = 0.5323$), from the third to fourth instar ($F_{1,35} = 0.34$;

$P = 0.5618$), from the fourth to fifth instar ($F_{1,35} = 0.26$; $P = 0.6110$), or from the fifth instar to adult ($F_{1,35} = 1.99$; $P = 0.1670$).

3.5 Discussion

Nosema maddoxi negatively impacted *H. halys* female survival when treated females were given the high spore concentration, but did not impact *H. halys* female survival when treated females were given the low spore concentration. The difference between these two bioassays was the increase in the number of spores ingested by treated females in the high spore concentration bioassay, which could explain why the higher spore concentration caused a significant impact. A study that tested five different *N. apis* spore doses on two different races of honeybees found that as the dose increased, mortality increased and the lifespan was shortened (Malone and Stefanovic, 1999), and these results support our findings.

The percent mortality of treated females in the low spore concentration bioassay that had low-intensity infections was highest (100%) when females died between 0-10 days after inoculation, and the percent mortality of treated females with high-intensity infections was highest (100%) when females died between 41-44 days after inoculation. A study on infection intensity of *N. ceranae* in honeybees (*A. mellifera*) determined that infection intensity was higher in older bees than in newly emerged bees (Jack et al., 2016), which could explain why, in our study, females that died sooner in the low spore concentration bioassay had low-intensity infections.

Not all treated adults in both spore concentration bioassays died before the end of the study. In the adult low spore concentration bioassay, 35.4% of the treated

females survived until the end of the study (42-44 days after inoculation) and 29.4% of the surviving treated females had low-intensity infections and 70.6% had high-intensity infections. In the high spore concentration bioassay, 24.1% of treated females survived until the end of the study (42-44 days after inoculation) and all had high-intensity infections. This indicates that a higher spore concentration does cause more mortality, but it did not cause mortality in all of its hosts within the 42-44 days after inoculation. It also shows that infection is variable between hosts. Some studies have shown that insect hosts could have varying levels of resistance or tolerance to microsporidian infections (Hoch et al., 2004; Vijendravarma et al., 2008; Hoch and Solter, 2018). This could explain why some treated females were able to live throughout the bioassays although they were infected and died with low- or high-intensity infections. Currently, there is no information about *H. halys*' immune responses to *N. maddoxi* infection, other than an inconsistent presence of tissue melanization in some field-collected *H. halys* that were infected (see Chapter 2).

Hopper and Mills (2016) determined that *N. fumiferanae postvittana* impacted immature survival, with only 26% of *E. postvittana* larvae surviving to adults. In our study, 56% of treated nymphs died before molting to the fourth instar, and only 26% eclosed into adults. Other studies have shown that early immature stages are usually more susceptible to microsporidian infections (Solter, 2014). We did not confirm whether *H. halys* nymphs were more susceptible than adults, but we can confirm that *H. halys* nymphs are susceptible to *N. maddoxi* infection.

Several studies on the impact that microsporidia have on immature stages of insects have indicated that they can prolong development and cause stunted growth

(Gupta et al., 2016; Hopper et al., 2016; Hoch and Solter, 2018). This was not observed in our study, indicating that *N. maddoxi* infection may not affect *H. halys* nymphal development rate and size. However, nymphs in the bioassay were given 28,000 spores and testing a higher spore concentration may produce different results.

In addition to shortening the lifespan of *H. halys* adults and nymphs, *N. maddoxi* also negatively impacted female fecundity and egg viability in both adult bioassays. Several microsporidian species have been known to impact female fecundity and egg viability, such as *N. fumiferanae postvittana* in *E. postvittana* (Hopper et al., 2016) and *N. bombycis* in *Bombyx mori* (Gupta et al., 2016). In the field, some microsporidian species that have been observed to cause negative impacts on female fecundity and egg viability in the lab have also impacted insect populations such as *Nosema pyrausta* in *Ostrinia nubilalis* (Siegel et al. 1986) and *P. locustae* in grasshopper populations in Saskatchewan, Canada (Ewen and Mukerji, 1980). Based on these studies, *N. maddoxi* could impact *H. halys* population densities in the US.

This study has demonstrated that *N. maddoxi* infection causes negative effects on *H. halys* in a laboratory setting. When the spore concentration was increased, *N. maddoxi* infection not only shortened the lifespan of *H. halys* females but reduced female fecundity and egg viability. In addition, *N. maddoxi* infection caused significant nymphal mortality, killing 74% of nymphs before they eclosed into adults when they were infected as second instars. Based on these results, we conclude that this native entomopathogen has the ability to negatively impact *H. halys* survival and overall fitness.

3.6 Acknowledgements

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