Ecology of the Brown Marmorated Stink Bug (Hemiptera: Pentatomidae): Oviposition on Different Trees, Nymphal Development on Various Vegetable Hosts, and Assessment of a Bacterial Pathogen

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Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is an invasive species that has spread throughout the United States and become a serious agricultural pest. Studies were conducted in Virginia on the survival of *H. halys* nymphs on different vegetable hosts and on the oviposition preference of *H. halys* on common tree hosts in southwestern Virginia. To determine the effects of vegetable host on *H. halys* survival, nymphs were placed on one of five vegetable host plants and their corresponding vegetable fruit. Survival was highest on sweet corn, followed by bell pepper and snap bean. Eggplant and tomato were poor compared to other vegetable hosts tested, potentially due to secondary compounds released by the plant to reduce feeding. Of six common host trees sampled, *H. halys* laid the most egg masses on catalpa, *Catalpa bignonioides*, while aggregation lure had no effect on the number of eggs laid. Across host trees, egg masses were more commonly found in trees with fruiting structures present. An additional study was conducted on a red-sternum phenotype of *H. halys*. Mortality was recorded by sternum phenotype for each sex. There was no difference in survival between groups at two time points. Biochemical tests were used to determine if the red-sternum was associated with the potentially pathogenic red bacteria *Serratia marcescens*. Only one stink bug's gut contents had a confirmed infection of *S. marcescens*. Results from these studies offer insight into *H. halys* development on vegetable hosts and oviposition preferences on different host trees.

General Audience Abstract

The brown marmorated stink bug (BMSB), is an invasive insect that has spread throughout the United States and become a serious pest. Current research is focused on reducing their populations without increasing insecticide use. Studies were conducted to determine if vegetable host type influenced survival and development of this insect. Nymphs were placed on one of five vegetable plants with the corresponding vegetable fruit. Overall, survival was highest on sweet corn, followed by bell pepper and snap bean. Eggplant and tomato were poor hosts, potentially due to chemicals released by the plant to reduce insect feeding. Six common host trees of BMSB were sampled to determine where females lay the most egg masses and the effect of using an attraction lure. Catalpa trees had the most egg masses throughout the season. Use of a lure had no effect on the number of eggs laid on any tree. A red colored abdomen was observed in some adult insects throughout these studies. To determine if this was associated with higher mortality, male and female insects were observed for two weeks. There were no mortality differences at either seven or 14 days between any of the groups. Tests were used to determine if the red abdomen was caused by a red-colored bacterium. Of the insects sampled, there was only one stink bug with a confirmed infection. Results from these studies provides information that increases knowledge of this pest's ecology in Virginia.

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Contributions of Authors

Writing was done by Adam Formella. All committee members assisted in planning the experiments, as well as making edits to the thesis. Dr. Thomas Kuhar was responsible for guidance throughout the course of the experiments and writing. He was also responsible for multiple levels of edits, both at the rough draft and second draft stages. Dr. Sally Taylor and Dr. Ann Stevens were both responsible for helping to plan the protocols of the experiments as well as making edits to the draft of the thesis before submission.

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Chapter 1

Literature Review

Halyomorpha halys (Stål)

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is an invasive insect pest. Reviews of its biology, ecology, and management in the U.S. can be found in Rice et al (2014) and Leskey and Nielsen (2017). In addition, Lee et al. (2013) provides an excellent review of much of the Asian literature on this insect. In this chapter, some of the literature that is pertinent to the research presented in this thesis is highlighted.

Distribution

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is native to east Asia (Lee et al. 2013). In the 1990s, the insect was detected in Allentown, PA (Hoebeke and Carter 2003) likely originating from the Beijing area of China with an estimated propagule size of only two to 18 females based on genetic markers (Xu et al. 2014). Since its arrival to the United States, *H. halys* has spread throughout much of the contiguous states and across Eastern and Western Canada (Rice et al. 2014, Leskey and Nielsen 2017). During this time, *H. halys* has also invaded almost a dozen countries in Europe including: Switzerland, Germany, France, Italy, Greece, Hungary, Romania, Spain, Georgia, Russia, and Abkhazia (Haye et al. 2015, Leskey and Nielsen 2017), and has recently become established in Chile (Leskey and Nielsen 2017). Invasive populations have mostly originated from China, with the exception of the populations in Italy, which were likely introduced by the U.S. (Valentin et al. 2017). Insights into *H. halys* biology help explain how the stink bug is capable of spreading to a variety of geographic areas.

Biology

Halyomorpha halys spend the winter months as a diapausing adult in sheltered locations such as dead standing trees in the woods (Lee et al. 2014), rock crevices, and inside buildings and homes where large aggregations can be found in attics or under siding or between walls (Inkley 2012). In the eastern U. S., adults emerge from overwintering sites as early as March and April (Nielsen and Hamilton 2009b, Bergh et al. 2017), but a lot is still unknown about their biology and movement immediately after overwintering (Leskey and Nielsen 2017). They are capable of long post-overwintering flight (Lee and Leskey 2015), but it is not known exactly where bugs go during the spring when suitable host plants are sparse.

Adult females require 148 degree days at a minimum temperature of 14.2 °C to finish development of reproductive organs, beginning once 12.7 hr. of daylight is reached (Nielsen et al. 2008; 2017). In the mid-Atlantic Region, *H. halys* eggs are not typically seen until the month of May. On average, females lay egg masses containing 28 eggs and can lay eight egg masses throughout their lives (Nielsen et al. 2008). In part, this large number of offspring per generation leads to rapid population growth. Eggs hatch after ~4 days at 25 °C. *Halyomorpha halys* has five nymphal instars. First instars remain on the egg mass to feed and acquire gut symbionts left from their mother (Nielsen et al. 2008, Taylor et al. 2014). The remaining four instars all feed on plants, with each instar slightly larger than the last (Hoebeke and Carter 2003). Development duration is largely dependent on temperature, with optimal development occurring around 25 °C and complete development requires ~44 days (Nielsen et al. 2008). With variation due to temperature and location, there are either one or two generations per year in the eastern U. S. (Nielsen and Hamilton 2009b). Around the time of the autumnal equinox (late September to October), sexually immature

adults seek overwintering sites, undergo diapause, and the cycle begins again (Nielsen and Hamilton 2009b).

The rapid spread of *H. halys* is attributed, in part, to its wide host range of over 170 plant species (Leskey and Nielsen 2017). In the U.S., *H. halys* has shown a preference for angiosperm trees (Bergmann et al. 2016). In the mid-Atlantic U.S., the invasive tree of heaven, *Alianthus altissima*, has become a dominant tree along wooded borders and disturbed areas, and has been shown to be an important tree for *H. halys* reproduction (Bakken et al. 2015, Martinson et al. 2016), and its subsequent movement to agricultural crops (Aigner et al. 2017). In the U.S., *H. halys* feeds on many cultivated plants including tree fruit, tree nuts, fruiting vegetables, as well as row crops such as corn, sorghum, soybean, and cotton (Kuhar et al. 2012, Leskey et al. 2012a, Pfieffer et al. 2012, Rice et al. 2014, Leskey and Nielsen 2017).

Feeding on agricultural crops can result in yield losses depending on the timing and severity of the feeding. In tree fruits, early season *H. halys* feeding can result in premature fruit fall in apples and peaches, while mid-season feeding can result in internal fruit damage (Nielsen and Hamilton 2009a). In 2010, *H. halys* caused an estimated $37 million in economic loss to apples alone (American/Western Fruit Grower 2011). Feeding injury is a result of the bug's stylets being inserted into the fruiting structure and dissolving internal tissue with its saliva and leaving behind a salivary sheath (Peiffer and Felton 2014). In turn, this feeding results in the fruiting structures being scarred externally, turning corky or spongy internally where feeding occurred, and can even lead to aborted fruit (Kuhar et al. 2012). Where the bug has become well established, feeding damage to certain crops can be substantial exceeding 50% in fruiting vegetables, and as much as 90% in other crops such as peaches and sweet corn (Leskey and Hamilton 2010, Kuhar et al. 2012, 2014, Rice et al. 2016). Additional research on the suitability of different host plants to support

H. halys development is needed. Chapters 2 and 3 of this thesis aim to provide further insight into this question.

Population Ecology of *H. halys*

Halyomorpha halys populations are regulated by abiotic as well as biotic factors. Temperature has a critical role in *H. halys* biology and will likely define the ultimate range of establishment of this species (Zhu et al. 2012). High temperatures (exceeding 30 °C) have been shown to negatively impact nymphal development and fecundity (Neilsen et al. 2008) as well as kill essential endosymbionts located on *H. halys* egg masses, which can lead to early instar mortality (Taylor et al. (2014). Further, exposures to temperatures exceeding 40 °C can be outright fatal to *H. halys* (Aigner and Kuhar 2016). Thus, *H. halys* will likely not flourish in extremely hot climates.

Adversely, *H. hays* is freeze intolerant and exposure to subfreezing temperatures can significantly reduce overwintering survival (Kiritani 2007, Cira et al. 2016). Thus, even though some *H. halys* find adequate shelter during the winter months, extreme cold events can reduce population levels the following spring (Cira et al. 2016).

Biotic factors can regulate *H. halys* populations as well. In its native region, *H. halys* populations are often maintained below damaging levels by natural enemies, especially scelionid egg parasitoids (Yang et al. 2015, Abram et al. 2017). One parasitoid species that plays a key role is *Trissolcus japonicus* (Hymenoptera: Scelionidae), which has been observed with parasitism rates as high as 80% in China (Yang et al. 2015).

As with many invasive species in North America, *H. halys* populations have flourished in the absence of key natural enemies (Wolfe 2002). Although a few native North American stink bug parasitoids will attack *H. halys* in its introduced range, these species either cannot successfully complete development on *H. halys* (Dieckhoff et al. 2017) or are generalists that are often habitat

specific (Herlihy et al. 2016). For example, in woodland habitats, *Anastatus spp*. (Hymenoptera: Eupelmidae) are the most prominent parasitoid of *H. halys*, although parasitism rates are generally low (Jones et al. 2017).

To combat this, research is focused on *T. japonicus,* which was originally brought to the United States in 2007, and has been maintained in quarantine ever since. However, adventive populations of this parasitoid have been found since 2014 (Talamas et al. 2015), and populations are spreading. Some labs have begun to mass rear *T. japonicus* and augmentatively release it in new areas (stopbmsb.org). Ideally, full establishment of this parasitoid species throughout the range of *H. halys* will help suppress population levels in the future.

Aside from parasitoids, bacteria and fungi may also provide natural control of *H. halys*. Hajek et al. (2017) recently identified the microsporidia *Nosema maddoxi* spp. from *H. halys* populations in the U.S. The entomopathogenic microbe is toxic to nymphs, with mortality of ~60% for second instars (Hajek et al. 2017). The bacteria *Serratia marscecens* has also been investigated. One strain of *S. marcescens* showed 100% lethality of *H. halys* in as little as two days and is currently under patent as a biocontrol agent (Martin et al. 2016). Research on pathogens as control agents of *H. halys* is just beginning and much work is needed in this area.

Serratia marcescens

Serratia marcescens is a gram negative rod in the family Enterobacteriaceae (Yu 1979). Bacterial colonies are red in appearance due to production of a red pigment, prodigiosin (Khanafari et al. 2006). Pigment production varies depending on a number of different factors including incubation time and growth substrate (Giri et al. 2004). Due to this bright red pigment, *S. marcescens* has been used throughout history, most notably in the 1950s where the U.S.

government spread the bacteria in an attempt to study nuclear drift (Tansey 2004). Unfortunately, this event lead to multiple illnesses and even death, as *S. marcescens* was discovered to be a human pathogen (Kreston 2015, Sharanya et al. 2018). Similarly, *S. marcescens* has been shown to be an entomopathogen, killing a number of insects including *Helicoverpa armigera, Phyllophaga blanchardi*, and *H. halys* (Mohan et al. 2011, Pineda-Castellanos et al. 2015, Martin et al. 2016). One potential factor for *S. marcescens* entomopathogenicity is its production of extracellular chitinases, which have been reported at concentrations higher than over 100 bacteria tested by Monreal and Reese (1969). It has also been shown that these chitinases work synergistically with the red prodigiosin pigment and may be indicative of pathogenic strains (Someya et al. 2001). Chapter 4 of this thesis focuses on natural *S. marcescens* in *H. halys* populations from Virginia.

Pest Management of *H. halys*

Chemical control has been the most widely used strategy for reducing *H. halys* damage to crops in the U.S. where this invasive species has established. This has resulted in an overall increase in insecticide usage. For instance, tree fruit growers in the mid-Atlantic U.S. increased the number of insecticide applications, in some cases nearly fourfold from 2010 to 2011 and reduced the interval between sprays (Leskey et al. 2012a). Active ingredients that have been most effective include pyrethroids (e.g., bifenthrin, permethrin, fenpropathrin, lambda-cyhalothrin, and beta-cyfluthrin), neonicotinoids (dinotefuran, clothianidin, imidacloprid, and thiamethoxam), carbamates (methomyl and oxamyl), and organophosphates (e.g., acephate and chlorpyrifos) (Kuhar and Kamminga 2017). All of these insecticides are broad-spectrum in their activity and will reduce natural enemy populations, potentially disrupting integrated pest management programs (Leskey et al. 2012a).

To return to an integrated approach to managing all pests in the crops affected by *H. halys*, a more sustainable strategy to supplement chemical control is needed. Two methods to reduce the volume of broad-spectrum insecticides used are the implementation of border sprays and action thresholds. The border spray technique involves spraying only the outer rows of a field (Trimble and Vickers 2000). Border sprays have been shown to be an effective management technique to help reduce *H. halys* damage in soybeans, apples, and peaches; in peaches, insecticide application has been reduced by as much as 61% (Blaauw et al. 2014, Joseph et al. 2014, Aigner et al. 2017). Threshold based control involves spraying crops once a population size is reached either in traps or on the crops themselves. Thresholds vary from crop to crop based off tolerance to damage and crop value (Zadoks 1985). For *H. halys*, threshold population sizes have been determined for apples at 10 adults and for peppers at five adults per weekly trap catch (Short et al. 2016, Bush et al. 2018).

Pheromone based lures and insect specific traps have been developed to help monitor *H. halys* populations and to assist with integrated pest management approaches. The first lure, a combination of stereoisomers (3S,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol and (3R,6S,7R,10S)-10,11-epoxy-1 bisabolen-3-ol, was developed from male *H. halys* and shows attraction of all life stages (Khrimian et al. 2014). This aggregation lure was improved upon in with the addition of methyl (E,E,Z)-2,4,6-decatrienoate to add a cross attraction to nymphs and adults season long (Weber et al. 2014). These lures were then added to trapping studies to develop specific traps for *H. halys*. Original *H. halys* traps were designed to mimic trees, with a dark pyramid base similar to a tree trunk, and a jar top with insecticidal strips to capture and kill the bugs (Leskey et al. 2012b). *Halyomorpha halys* traps were modified by adding sticky cards to stakes to reduce check times, while being correlated to the previous trap type so thresholds in place could be adjusted

accordingly (Rice et al. 2018). The combination of pheromone lures and insect specific traps has been critical in monitoring and developing thresholds.

Gaps in the Literature

Since *H. halys* introduction to the U.S. in the 1990s, many research questions have been answered about its biology, ecology, and management. There are still many areas that need to be explored, particularly when it comes to integrated pest management practices for the pest. The research in this thesis focuses on gathering more information on *H. halys* ecology in Virginia. In this study, *H. halys* development and survival was measured on various vegetable crops. Prior work has been done only in tree fruit systems with peaches and apples and little is known about *H. halys* develops on vegetables (Acebes-Doria et al. 2016). It is not known when and where *H. halys* lays its eggs in an urban forest environment. This knowledge is crucial to optimize potential releases of *T. japonicus*. Finally, the relationship between *S. marcescens* and *H. halys* needs to be explored to determine if there is a correlation of a red-sternum phenotype with higher mortality in *H. halys* adults.

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Chapter 2

Halyomorpha halys (Hemiptera: Pentatomidae) nymphal development on different vegetables

Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is an invasive stink bug originating from eastern Asia. Since its arrival to the United States, *H. halys* has become a serious economic pest to a wide variety of crops. Some of these crops include vegetables, where the bug causes injury by feeding on the vegetable fruit, resulting in discoloration and reduced marketability. To further our understanding of *H. halys* pest status in vegetables, a laboratory study was conducted to determine if vegetable host type affects the development of nymphs. Tomato, sweet corn, eggplant, bell peppers, and snap beans were evaluated. Egg masses containing second instars were caged with a single vegetable plant and its corresponding fruit. Survival and development of nymphs were tracked until adulthood. Five plants of each vegetable type were used. This experiment was replicated nine times over two years. Survival and development of nymphs differed by host type. The highest survival was observed on sweet corn and the lowest survival on eggplant and tomato. Results of this study help increase our understanding of how *H. halys* interacts with various vegetable crops.

Introduction

Halyomorpha halys (Stål) is an invasive pest originating from the Beijing area of China. It was first detected in the United States in the 1990s in Allentown, Pennsylvania (Hoebeke and Carter 2003, Xu et al. 2014). Since its arrival, *H. halys* has spread throughout much of the United

States becoming a serious pest of a number of economically important plants (Rice et al. 2014, Leskey and Nielsen 2017). Fields of sweet corn, bell peppers, tomatoes, eggplants, and snap beans have been frequently infested and damaged by this pest in the mid-Atlantic region (Kuhar et al. 2012b, Cissel et al. 2015, Zobel et al. 2016, Philips et al. 2017). *Halyomorpha halys* feeding on these vegetables may cause conspicuous white or yellow feeding marks on the external surface of the fruit, soft or spongy areas in the fruit where feeding occurred, as well as increased rotting and aborted fruit (Kuhar et al. 2012b). On sweet corn, *H. halys* feed directly through the ear husk injuring individual kernels (Cissel et al. 2015). This polyphagous insect feeds on a wide range of plants (Bergman et al. 2016, Bakken et al. 2015, Leskey and Nielsen 2017) and can be successfully reared in the laboratory using a mixed diet of various seeds, nuts, beans, or carrots (Medal et al. 2012, Beatrice and Jackai 2017). There is little information on how different vegetable host plants support development of *H. halys* in the wild. Host type influences survival and development of nymphs in other systems. In tree fruit, Acebes-Doria et al. (2016) showed that *H. halys* nymphs survive and develop better on leaves and fruit of peaches than apples, and that nymphs develop best when provided with multiple hosts over an individual host.

Halyomorpha halys can complete development from egg to adult in 537.63 degree-days at a minimum and maximum temperature of T_o = 13.94 and T_m = 35.76, and is univoltine in most of the mid-Atlantic region (Nielsen et al. 2008). Their life cycle includes five nymphal instars, the first of which remain on the egg mass feeding on the chorion to obtain symbiotic bacteria (Taylor et al. 2014). After molting, second instars leave the egg mass in search of food (Rice et al. 2014). The host plant(s) available at this time can be critical to the developmental success and survival of *H. halys*.

Here, we report the results of a laboratory study assessing the survival and development rate of *H. halys* nymphs on five different vegetables: bell peppers, snap beans, eggplants, tomatoes, and sweet corn. Methods were adapted from Acebes-Doria et al. (2016) where nymphal development and survival were observed from second instar to adult on leaves and fruit of different trees. We hypothesized that survival and development time will be affected by vegetable food source.

Materials and Methods

Insects. Wild *H. halys* egg masses were collected from various tree species, including catalpa, *Catalpa bignonioides*, and redbud, *Cercis canadensis*, in Blacksburg, Virginia in July and August of 2017 and in June of 2018 by removing leaves containing egg masses. The number of eggs per egg mass as well as the tree species and location was recorded for each egg mass. Egg masses were transferred to individual 60 × 15 mm Petri dishes (Fisher Scientific International Inc., Hampton, NH) and were placed in a growth chamber set to 26 °C, 70% RH, and a photoperiod of 16:8 (L:D) h. A moist water wick was placed with egg masses to prevent desiccation of the eggs and to provide water for the hatched nymphs. Egg masses were kept in the growth chambers until hatched nymphs molted into second instars, at which time, they were placed on each vegetable species. Only newly-molted second instars of approximately the same age were used. One egg mass was used per vegetable development cage.

Vegetable Development Cages. One vegetable plant with its corresponding fruit and a water wick were placed into a 30.5-cm^3 screened cage (BioQuip Products Inc., Rancho Dominguez, CA). Vegetables tested in this experiment included: bell pepper (*Capsicum annuum*, variety 'Aristotle'), snap bean (*Phaseolus vulgaris*, variety 'Caprice'), eggplant (*Solanum*

melongena, variety 'Classic'), tomato (*Solanum lycopersicon*, variety 'Baby cakes'), and sweet corn (*Zea mays saccharata*, variety 'Jubilee'), with tomato variety 'Mountain Fresh Plus' used in 2018 due to availability. One of each vegetable type was tested in each replication of the experiment, with a total of nine replicates conducted. Seeds were planted and plants were grown in a greenhouse for three to four weeks until they were transferred to cages. The fruit of each of the vegetable species were collected from field plants grown at Kentland Farm in Whitethorne, VA. When fruit were not available in the field, organically grown fruit were purchased from local markets. All fruit were washed with water to help remove materials that might be hazardous to the *H. halys* nymphs (e.g., insecticides, fungicides, fungal spores, microbes). Vegetable fruit were provided to fill the lid of a 60 × 15 mm Petri dish for each development cage. Fruit too large to fit in the lid were cut and placed with the cut surface down and unexposed as wild nymphs would not come in contact with these portions of the fruit. For corn, the kernels and husk were kept on the cob, but the cob was cut to size to fit into the Petri dish lid. In addition to vegetable plants and fruit, water was provided in a 30 ml soufflé cup with a cotton wick inserted through the lid. Plants, fruit, and water were checked every 48 h and replaced as needed. Cages were kept in a rearing room maintained at 26.0 ± 1 °C, with a relative humidity of 56.0 ± 1%. Light was provided by 50W plant bulbs hung 0.5 m above the cages programmed to 16:8 (L:D) h.

Seven replicates of an experiment were run between July 2017 and October 2018 and an additional two replicates between June 2018 and August 2018. Within each replicate, vegetable development cages were started within 48 h, due to the nature of collecting wild egg masses, as multiple egg masses needed to be collected to assure they would all molt into second instar at the same approximate time. Freshly molted 2nd instar *H. halys* were kept with their egg mass, containing at least 20 nymphs (M = 23.8, sd = 2.94), and were randomly assigned to an individual

cage. Second instars were used because first instars do not feed on plant tissue and will often die before molting to second instar if removed from their egg mass (Acebes-Doria et al. 2016, Beatrice and Jackai 2017). Deceased *H. halys* and exuvia were removed from the cages to reduce the chance of disease.

Data Collection and Analysis. The number of live and dead nymphs and their instar were recorded every two to three days until the entire cohort completed development to adulthood or died. Temperature and relative humidity were monitored continuously using a data logger (HOBO U10 Temperature and Humidity Data Logger, Onset Computer Corporation, Bourne, MA). Egg mass generation was taken into account based on egg collecting period. Eggs collected in June and July were considered early egg masses as they were laid by overwintering adults (Chapter 3), while eggs laid in August were categorized as late eggs. The interaction between egg generation and vegetable host on survival and developmental duration were analyzed using a two-way analysis of variance (ANOVA). Wilcoxon each pair test was used to generate a connecting letters report for the vegetable hosts at each life stage. Data were analyzed using JMP® Pro version 13 (SAS Institute Inc., Cary, NC, 2007) where significance was determined by alpha < 0.05.

Results

There was a significant interaction effect between egg mass generation and vegetable host on survival for all instars, except at development from 2nd to 3rd instars (Table 2.1). There was no effect of interaction between egg mass generation and developmental duration on vegetable hosts for any of the instars (Table 2.1).

Due to the interaction effect between egg mass generation and vegetable host on *H. halys* survival (Table 2.1), the effects of vegetable host were analyzed separately by egg mass generation (Figure 2.1). There was no effect of vegetable host on survival between any instar from egg masses

collected from the early summer, potentially due to low overall survival (Figure 2.1). In contrast, there was an effect of vegetable host on all instars from egg masses collected in the late summer.

For the egg masses collected from the late summer, survival to the 3rd instar was lowest on tomato, compared to pepper, eggplant, and corn, but not when compared to snap bean. Survival to the 4th instar was lower for tomato compared to sweet corn and pepper, but there were no differences between eggplant and snap bean nor between snap bean, bell pepper, and eggplant or between sweet corn, eggplant and pepper. Survival to the 5th instar was lower in eggplant and tomato compared to pepper, but there were no differences between sweet corn, snap beans, and bell pepper nor between eggplant, snap bean, and sweet corn. Survival to the adult stage was lower in eggplant and tomato compared to pepper, but there were no differences between corn, snap beans, and bell pepper nor between eggplant, sweet corn, and snap bean. These results are summarized in (Figure 2.1).

There was a significant effect of food source on nymphal development rate from 2nd to 3rd instar ($F = 4.91$; $df = 4, 31$; $P \leq 0.004$) with development occurring the fastest on snap bean (9.7 ± 0.9 d) and sweet corn (10.9 ± 1.0 d) and the slowest on tomato (17 ± 2.0 d; Table 2.2). Developmental rates were similar on all vegetables from 3rd to 4th instar ($F = 1.43$; $df = 4, 27$; $P = 0.255$), and from 4th to 5th instar ($F = 2.29$; $df = 4, 22$; $P = 0.099$; Table 2.2). However, there was a significant treatment effect on developmental time from 5th instar to adult ($F = 3.06$; $df = 4, 21$; $P \leq 0.046$), and overall from 2nd instar to adult ($F = 3.08$; $df = 4, 21$; $P \leq 0.044$) with development time being the shortest on sweet corn and longest on bell pepper (Table 2.2).

Survival and development duration results showed variability due to high mortality in various cohorts. Of the 49 cohorts tested, 24 did not develop to adulthood, with at least one cohort from each vegetable not completing development. This was especially true for tomato where seven

of the nine cohorts died before molting into third instar and only five individuals survived to adulthood. Other factors that may have affected survival and development duration, such as temperature and relative humidity, were tested to determine if there was a difference across the time it took each cohort to develop. No significant difference in the average temperature or the average relative humidity was found and means were within 1 °C and 1% RH.

Table 2.1. Two-way ANOVA reported statistical values for *H. halys* survival and development duration analyzed by vegetable host, egg mass generation, and the interaction of the two on each developmental stage from 3rd instar to adult.

		Dev. to 3rd			Dev. to 4th			Dev. to 5th			Dev. to Adult		
Location	Source	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*
Survival	Vegetable (V)	4.349	4, 44	0.0058	5.982	4, 44	0.0009	6.139	4, 44	0.0007	7.011	4, 44	0.0003
	Generation (G)	16.450	1, 44	0.0003	18.784	1, 44	0.0001	14.095	1, 44	0.0006	14.431	1, 44	0.0006
	V × G	2.381	4, 44	0.0703	3.301	4, 44	0.0214	3.039	4, 44	0.0299	2.900	4, 44	0.0357
Developmental Duration	Vegetable (V)	7.218	4, 31	0.0007	1.553	4, 27	0.2296	1.0251	3, 22	0.4113	0.531	3, 21	0.6690
	Generation (G)	3.941	1, 31	0.0597	0.034	1, 27	0.8565	---*	---*	---*	---*	---*	---*
	V × G	2.074	4, 31	0.1188	1.530	4, 27	0.2358	0.425	3, 22	0.7382	0.783	3, 21	0.5243

* No value reported due to few or no nymphs completing development and no degrees of freedom

Table 2.2. Percent mean development duration in days ± standard error on each vegetable type at every developmental stage from 3rd instar to adult as well as the total developmental duration and numbers of *H. halys* completing development.

Vegetable (N)[a]	Mean ± SE days				Total Duration (N)[b]
	Third	Fourth	Fifth	Adult	
Snap bean (221)	9.6 ± 0.9a	12.5 ± 1.0b	11.0 ± 1.5a	16.4 ± 2.1ab	49.2 ± 3.3ab (44)
Bell pepper (212)	12.0 ± 1.1ab	11.6 ± 1.1ab	14.7 ± 1.6a	23.2 ± 2.4b	61.5 ± 3.7b (41)
Eggplant (220)	14.4 ± 1.0b	12.5 ± 1.1ab	14.6 ± 1.5a	16.2 ± 2.4ab	53.2 ± 3.7ab (6)
Tomato (210)	17.0 ± 1.9b	12.5 ± 2.0ab	8.0 ± 2.4a	11.0 ± 2.2ab	48.5 ± 5.3ab (5)
Sweet corn (212)	10.8 ± 0.9a	9.4 ± 1.0a	11.1 ± 1.2a	14.1 ± 1.8a	45.4 ± 2.8a (75)
One-Way ANOVA	$F_{4, 31} = 4.91$	$F_{4, 27} = 1.43$	$F_{4, 22} = 2.29$	$F_{4, 21} = 3.06$	$F_{4, 21} = 3.08$
	P = 0.004	P = 0.255	P = 0.099	P = 0.046	P = 0.044

[a] Total number of second instar for each vegetable.

[b] Total number of nymphs that developed to adult.

Means within the same column with a letter in common are not significantly different (P>0.05).

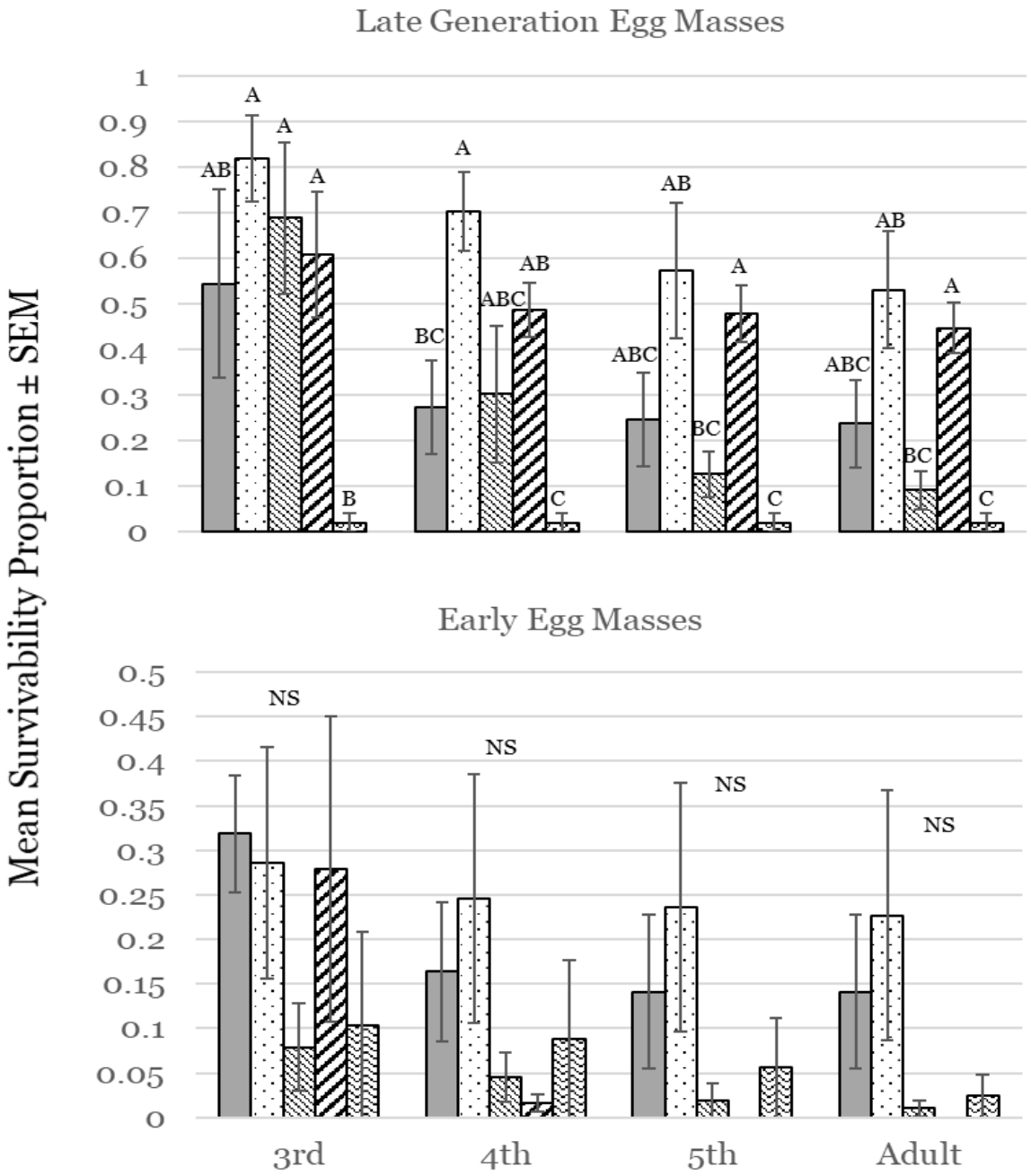

Figure 2.1. Effect of vegetable host on *H. halys* survival. Vegetables from left to right for each developmental stage are: bean, corn, eggplant, pepper, and tomato. Graphs are broken up by egg mass generation and show mean proportion surviving to the instar. Means with the same letter in the same development group (e.g. 3rd, 4th, 5th,) are not significantly different ($P > 0.05$). Letters NS indicate no significant difference between means.

Discussion

Results from this study show an increase in fitness in egg masses from late summer versus early summer egg masses. This trend was observed in survival of *H. halys* nymphs from early summer egg masses where the highest mean survival to adults was in corn at 23% versus 53% survival to adulthood in corn from late summer egg masses (Figure 2.1). This apparent increase to nymphal fitness was also observed in the types of vegetables that *H. halys* could complete development on. In egg masses collected from early summer, survival was low on pepper; however, in late summer eggs, survival was highest on pepper, although not significantly different than all other vegetable hosts. These findings are critical to developments of future studies dealing with wild collected *H. halys* egg masses, as survival and fitness seem to be affected by collection timing.

Halyomorpha halys nymphs survived and developed best on sweet corn (36% completing development), followed by pepper and snap bean (18-20%); eggplant and tomato proved to be poor host species for *H. halys* with <5% of nymphs completing their development on those vegetables. Our results may help explain why sweet corn often has a high incidence of *H. halys* nymphs and typically higher than other vegetables (Kuhar et al. 2012b, Zobel et al. 2016). *H. halys* also developed well on snap beans and bell pepper, two crops that are also commonly attacked in the field (Kuhar et al. 2012b, 2014, Kuhar and Doughty 2016). This also supports the use of snap beans to feed lab colonies, a common method in many studies (Nielsen et al. 2008, Medal et al. 2012, Beatrice and Jackai 2017).

Eggplant and tomato were not suitable hosts for *H. halys* to complete development. This may be indicative of these plants either not having proper nutrients for *H. halys* development or from possessing secondary (defensive) plant compounds, which result in development difficulties

and mortality (War et al. 2012). In developmental cages with eggplants, lower survival was not observed until later instars potentially indicating an accumulation of defensive compounds. For tomato, this low survival was observed immediately with development from second to third instars. Zobel et al. (2016) also suggested that tomato is a poor reproductive host for *H. hays* after observing relatively few egg masses and nymphs on tomatoes in a mixed planting of several vegetable crops in Maryland. However, this does not mean that tomatoes do not incur stink bug feeding injury. On the contrary, high percentages of tomato fruit (exceeding 50% in some cases) may be injured by *H. halys* and other stink bugs each year in the mid-Atlantic U.S. (Kuhar et al. 2012a,b, Aigner et a. 2015, Rice et al. 2016, Zobel et al. 2016).

Nymphal development duration from 2nd instar to adult on suitable vegetable hosts such as sweet corn or snap bean alone required about 45 to 50 days, which is similar to what Acebes-Doria et al. (2016) observed on a single diet of peach (*H. halys* nymphs required 53.5 ± 2.7 days at 25 °C to develop). However, as has been noted previously, *H. halys* develop best on a mixed diet (Acebes-Doria et al. 2016, Beatrice and Jackai 2017, Leskey and Nielsen 2017). On a mixed diet of green beans and corn, Nielsen et al. (2008) determined that *H. halys* nymphs required about 33 days at a similar temperature of 25°C, to develop from second instar to adult. Thus, although our research findings have relevance to explaining apparent preferences of *H. halys* for certain vegetables over others in the field, these insects more often than not move from host to host as was reported by Zobel et al. (2016) and, in so doing, improve their fitness. The nutritional quality of plants is variable in space and in time and most stink bugs have adapted to explore alternate food plants during their development (Panizzi 2000).

Our results also may help explain why *H. halys* is particularly damaging on small multi-crop vegetable farms, particularly organic farms with numerous host plants (Mathews et al. 2017).

In contrast, monocultures of vegetables that *H. halys* does not develop well on, such as tomato and eggplant, would probably not have high populations of bugs, except perhaps on the edges of fields where wooded borders or other weeds or crops may be present.

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Chapter 3

Effects of aggregation lure and tree species on *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) seasonal oviposition

Abstract

The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål), is a polyphagous pest that feeds on a wide variety of agricultural commodities including tree fruits, berries, vegetables, and field crops. Accurate knowledge of where *H. halys* lays eggs is critical to optimize the potential release of *Trissolcus japonicus*, (Ashmead), a scelionid egg parasitoid native to the same host region as *H. halys*. Ideally, parasitoids should be released in and around areas with high host density. In southwestern Virginia, we searched trees for egg masses in an urban environment and in a non-managed wooded border environment. We also evaluated the effects of a commercial aggregation lure on the number of eggs being deposited. This aggregation lure, when combined with methyl (E,E,Z)-2,4,6-decatrienoate (MDT), has been shown to attract both adult and nymph *H. halys*, but its effects on egg laying has not been fully studied. Results of this study showed no effect from the lure on the number of eggs laid. Catalpa trees, *Catalpa bignonioides*, had the most egg masses throughout the course of the study; however, other tree species such as redbud, *Cercis Canadensis*, had similar numbers in the late July and August. There was an overall trend toward oviposition on trees with fruiting structures present. This information provides insight on where and when to make augmentative releases of egg parasitoids for *H. halys*.

Introduction

Native to East Asia, *Halyomorpha halys* (Stål) is an invasive stink bug pest that is rapidly spreading throughout North America and Europe (Haye et al. 2015, Leskey and Nielsen 2017). Since its arrival in the U.S. in the 1990s (Hoebeke and Carter 2003), from a population likely originating from Beijing, China (Xu et al. 2014), *H. halys* has caused millions of dollars of economic loss to a variety of commodities including tree fruit, vegetables and row crops (Leskey et al. 2012, Rice et al. 2014).

The accelerated rate of their spread and the extent of the economic damage they cause is due, in part, to their large host range of over 170 plants (Leskey and Nielsen 2017). Being able to feed on a variety of hosts enables *H. halys* to move from one plant to the next as fruiting structures and more suitable food become available (Zobel et al. 2016). Knowing what plants *H. halys* is primarily feeding and ovipositing on in the landscape is useful for monitoring and developing crop-specific scouting plans in new regions (Bakken et al. 2015). This is true for rural as well as urban and suburban landscapes as high numbers of *H. halys* adults seek shelter in human dwellings and buildings, then move to suitable host plants in the spring to begin feeding and reproduction (Bergmann et al. 2016).

In this study, we surveyed host trees in Montgomery County, Virginia that *H. halys* may deposit eggs on over its reproductive period (May – late Aug/early Sep). Tree species were chosen based on a prior study exploring the range of tree hosts in this area (Bakken et al. 2015) and the predominance of each species in the landscape. Also, to determine if these ovipositional trends could be manipulated by semiochemicals, commercial *H. halys* aggregation pheromone lures were added to half of the trees. Lures were comprised of a combination of stereoisomers (3S,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol and (3R,6S,7R,10S)-10,11-epoxy-1 bisabolen-3-

ol (Khrimian et al. 2014) that work synergistically with methyl (E,E,Z)-2,4,6-decatrienoate to attract both *H. halys* adults and nymphs (Weber et al. 2014, 2017). It is not known whether the lures influence *H. halys* oviposition preference. The results of this study will be pertinent to current biological control efforts for *H. halys*, in particular, surveys to detect adventive populations of the egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae). This species is capable of parasitizing *H. halys* egg masses in its native range at rates of almost 80% (Yang et al. 2015). This parasitoid has been detected in several states in the U.S. (Talamas et al. 2015, Herlihy et al. 2016, Hedstrom et al. 2017, Hoelmer and Tatman 2017), and efforts to re-distribute field-collected and lab-reared *T. japonicus* are in progress at the time of this manuscript. Thus, knowledge of where and when *H. halys* distributes most of its eggs would be particularly useful to this effort.

Materials and Methods

Trees and Selection Sites. In 2017 and 2018**,** known host plants (trees) were sampled for *H. halys* egg masses, nymphs, and adults. Host plants were chosen from Bakken et al. (2015), a study that sampled non-managed woodlands in Virginia and North Carolina for *H. halys* eggs, nymphs and adults. These plants include: *Catalpa bignonioides* (southern catalpa), *Acer rubrum* (red maple), *Magnolia grandiflora* (southern magnolia), *Amelanchier spp.* (serviceberry), *Cercis Canadensis* (eastern redbud), and *Cladrastis kentukea* (yellowwood), with yellowwood being added to the study in 2018 due to large amounts of *H. halys* found on this species in 2017. Previously existing trees were selected in urban and urban-bordering environments in the town of Blacksburg, Virginia. Sites were distributed over a 5.0 km radius centered at the intersection of Prices Fork Rd and Plantation Rd in Blacksburg, VA. At each site, only two of each host tree

species were selected. Qualifying trees were required to be at least 15 cm in diameter at 1.5 m height, have abundant foliage at 2 m height, and have a paired tree (i.e., a tree of the same species and phenotype) at least 50 m away, but within a 300 m radius (Fig. 3.1). Only two tree sites did not fit this description, a catalpa site in 2017 and a magnolia site in 2018. At these sites, trees were selected to match the phenology at the closest available location, 3.0 km for the catalpa trees and 4.0 km for the magnolia. Five separate sites were selected for each tree species. At each site, starting in May and being replaced every two months, a *H. halys* Dual aggregation lure (Trécé Pherocon, Adair, OK) was added to one tree with a binder or a paper clip connecting the lure to the tree at 2 m (Fig. 3.2), while the paired tree did not have a lure added. The same sites and trees were used in both years unless trees were diseased or damaged. To remedy this, sites/trees were changed to be as close as possible to the previous site/tree. In 2018, the tree containing the lure was switched in case one tree naturally attracted more *H. halys*. In total, 20 sites were sampled in 2017, and 26 sites in 2018.

Sampling Method. Trees were sampled every seven days to reduce the opportunity for egg masses to be laid, hatch, and develop into second instar (the stage at which *H. halys* nymphs move from the egg mass) between sampling visits. Trees were sampled by conducting visual counts for two minutes, similar to methods used in Bergmann et al. (2016). During these two minutes, *H. halys* egg masses, nymphs, and adults were counted as well as other stink bug egg masses, nymphs, and adults (Fig. 3.3). Because development from egg to second instar can occur in as little as six to seven days, first and second instar *H. halys* located on an egg mass were counted as an egg mass (Nielsen et al. 2008). All egg masses were collected and placed in individual 60 × 15 mm Petri dishes (Fisher Scientific International Inc., Hampton, NH) and placed in a growth chamber set to 26 °C, 70% RH, and a photoperiod of 16:8 (L:D). Egg masses were monitored

every 48 h and the number of hatched, unhatched, and parasitized eggs were recorded. Other stink bug egg masses were identified by clutch size, color and shape of eggs, and eggs were reared and nymphs/ adults were identified. Collection lasted from May until few (<2) or no egg masses were found which was September 12th in 2017 and August 28th in 2018.

Data Analysis. Data were analyzed using JMP® Pro version 13 (SAS Institute Inc., Cary, NC, 2007). Significance was determined at alpha < 0.05 across all tests. Data from 2017 and 2018 were analyzed separately. Repeated measures ANOVA was used to compare the effects of lure, tree species and time (month) on the number of *H. halys* eggs laid in both years. Effect of tree species was compared for months June, July, and August using Wilcoxon multiple comparisons test. The effect of site was not analyzed for this experiment as not all sites contained all tree species and were limited by the host trees already planted at the time of the start of the experiment. Trees were spread across multiple sites to reduce extraneous factors that were not possible to account for (e.g. sunlight, surrounding trees, human structures, rainfall differences).

Results

In both 2017 and 2018, *H. halys* was the predominant stink bug egg mass found (N = 66 and N = 122) in the trees sampled around Blacksburg, Virginia accounting for 72% to 85% of all egg masses, in 2017 and 2018, respectively (Fig. 3.6) The next most common stink bug species in all trees included dusky stink bug, *Euschistus tristigmus* (Say), and the rough stink bug *Brochymena quadripustulata* (Fabr.). *H. halys* made up over 50% of the egg masses found on all trees except red maple, 28% in 2017 and 42% in 2018 (Figs. 3.8 - 3.10). Catalpa had the second lowest percentage of non-*H. halys* stink bugs in 2017 (7%) and the lowest percentage in 2018 (6

%). Alternatively, red maple had the highest variety of stink bug egg masses, with 6 species found in 2017 and 5 species in 2018.

Over the course of the two-year study, 3,150 2-minute observations were conducted on trees around Blacksburg, Virginia, resulting in the finding of over 6,000 *H. halys* eggs from more than 180 egg masses. Of these eggs, 5,362 nymphs emerged with a hatch rate of 83.4% in 2017 and 95.0% in 2018. The remaining eggs were either unhatched (5.4% and 2.8%, in 2017 and 2018, respectively) or had parasitoids emerge. In both years, only two adult parasitoid species emerged, *Anastatus reduvii* (Howard) (Hymenoptera: Eupelmidae) and *Anastatus mirabilis* (Walsh and Riley) with *A. reduvii* being the predominant parasitoid, emerging from 265 eggs in 2017 and 65 eggs in 2018 versus *A. mirabilis* emerging from 74 and 2, respectively.

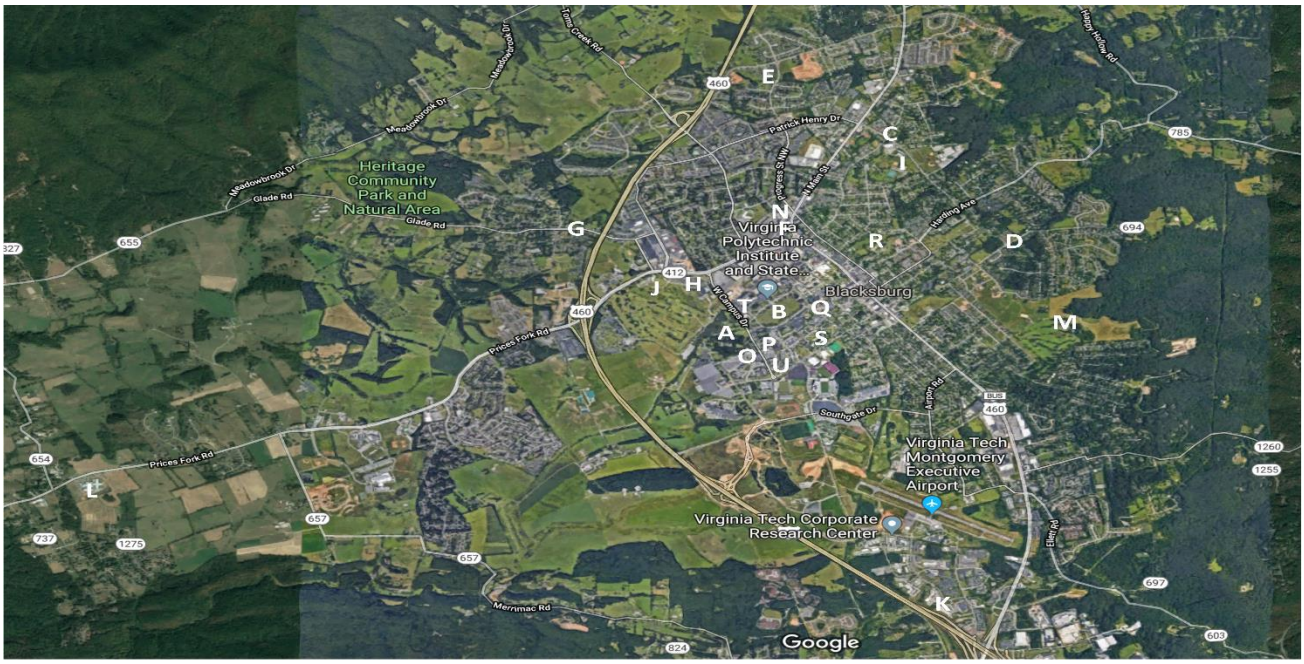
Repeated measures ANOVA showed no effect of lure on *H. halys* egg densities in 2017 ($P = 0.4492$) or 2018 ($P = 0.4383$). There was an effect of lure on the number of *H. halys* adults observed in both years ($P = 0.0128$ and $P = 0.0007$). Because there was no effect of lure on the number of egg masses found, data were combined to compare the effects of tree species by month (Fig. 3.4).

There was a significant effect of tree species ($P = 0.0025$ in 2017 and $P = 0.0102$ in 2018) and time ($P = 0.0059$) in 2018, but not in 2017 ($P=0.4841$) on *H. halys* egg densities. In 2017, catalpa trees had significantly more *H. halys* egg masses than any of the other tree species in June and July ($F = 2.95$; $df = 4, 45$; $P = 0.003$ and $F = 6.72$; $df = 4, 45$; $P = 0.0002$, respectively), and numerically more egg masses in August, although not significantly different from redbud, red maple or magnolia ($F = 2.34$; $df = 4,45$; $P = 0.069$; Fig. 3.5). In 2018, catalpa again had the highest number of *H. halys* egg masses in June ($F = 3.39$; $df = 5, 54$; $P = 0.0097$), significantly more than red maple or magnolia. In July 2018, red bud and catalpa had the most eggs, and in August, overall

densities of *H. halys* eggs were low and there was no significant effect of tree species ($F = 1.12$; $df = 5, 54$; $P = 0.3617$). In both years, serviceberry produced no egg masses in the month of August.

In addition to analyzing seasonal egg densities, another barometer to compare trees is to assess the percentage of trees within a species that had *H. halys* eggs. Across years and lure treatments, *H. halys* eggs were found on 90% of the catalpa trees sampled. In comparison, 65%, 60%, 45%, 35%, and 25% of trees had *H. halys* eggs for red bud, yellowwood, magnolia, red maple, and serviceberry, respectively.

In 2017, the peak timing for *H. halys* egg masses was mid-July, with another peak at the end of August (Fig. 3.6). Peak *H. halys* egg laying was a month earlier in 2018, with a peak in mid-June and another small peak again in August. In both years, a baseline of approximately four egg masses per 2-minute search per week (across all tree species) was found outside of the peak weeks. A peak of 2nd instars typically followed within two weeks of the peak egg laying in both years. This trend follows with peaks of subsequent instars following roughly two weeks after the previous life stage (Fig. 3.7). Ultimately, this resulted in a culmination of *H. halys'* life stages in mid-August in 2017 and early July in 2018.



Location	Key	Tree Species	Year
Duckpond	A	1 Catalpa and 1 Redbud	2017 & 2018
Drillfield	B	1 Catalpa, 1 Serviceberry, and 2 Yellowwood	2017 & 2028
Rutherford Town Homes Complex	C	2 Catalpa and 2 Redbud	2017 & 2018
Corner of Piedmont St. and Clay St.	D	2 Catalpa	2017 & 2018
Givens Lane	E	1 Catalpa	2017 & 2018
India Garden	F	1 Catalpa and 2 Redbud	2017 & 2018
Glade Road	G	2 Catalpa and 1 Magnolia	2017 & 2018
Alumni Center	H	2 Redbud, 2 Yellowwood, and 2 Magnolia	2017 & 2018
Aquatic Center	I	2 Redbud, 2 Red Maple, and 2 Serviceberry	2017 & 2018
Visitor Center	J	2 Redbud, 2 Serviceberry, and 2 Yellowwood	2017 & 2018
CRC Pond	K	2 Redbud, 2 Red Maple, and 2 Serviceberry	2017 & 2018
Prices Fork Elementary	L	2 Redbud, 2 Red Maple, and 2 Serviceberry	2017 & 2018
Blacksburg Golf Course	M	2 Red Maple	2017 & 2018
True Value	N	2 Red Maple	2017 & 2018
Hillcrest	O	2 Magnolia	2017 & 2018
Fralin	P	2 Magnolia	2017 & 2018
Graduate Life Center	Q	2 Magnolia	2017 & 2018
Blacksburg Cemetery	R	1 Magnolia	2017 & 2018
Basketball Practice Arena	S	1 Magnolia and 1 Serviceberry	2018
North Hahn	T	2 Yellowwood	2018
New Hall	U	2 Yellowwood	2018

Figure 3.1. Tree species location around Blacksburg, Virginia in 2017 and 2018 where weekly visual sampling was conducted for stink bugs. Trees are not arranged by site, but by location of the trees, as paired trees occasionally were not at the same named site.

Figure 3.2. A brown marmorated stink bug aggregation lure (Dual Lure) attached to a redbud branch with a paperclip at 2m directly under the foliage.

Figure 3.3. *Halyomorpha halys* adults feeding on serviceberry fruit in early June (left), and an *H. halys* egg mass with 1st instars on a catalpa leaf (right).

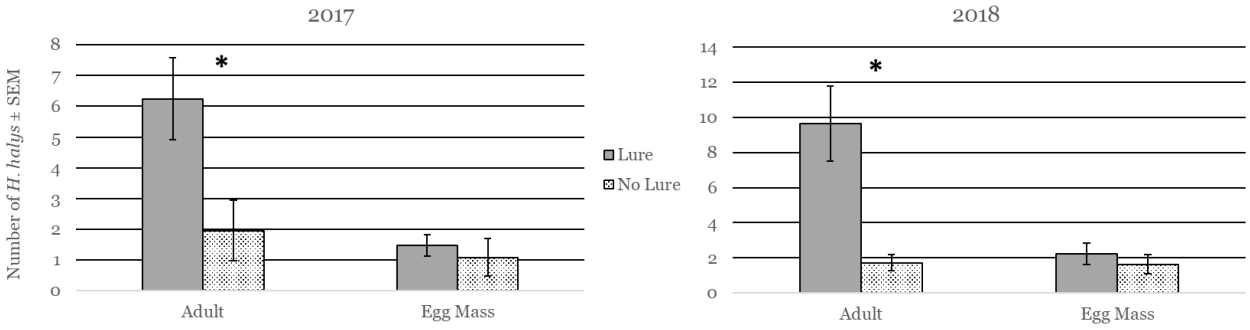


Figure 3.4. Effect of aggregation lure (Dual Lure) on *H. halys* adults and egg masses. Bars show cumulative counts on all tree species throughout the season. Asterisk indicates significantly different means ($P < 0.05$).

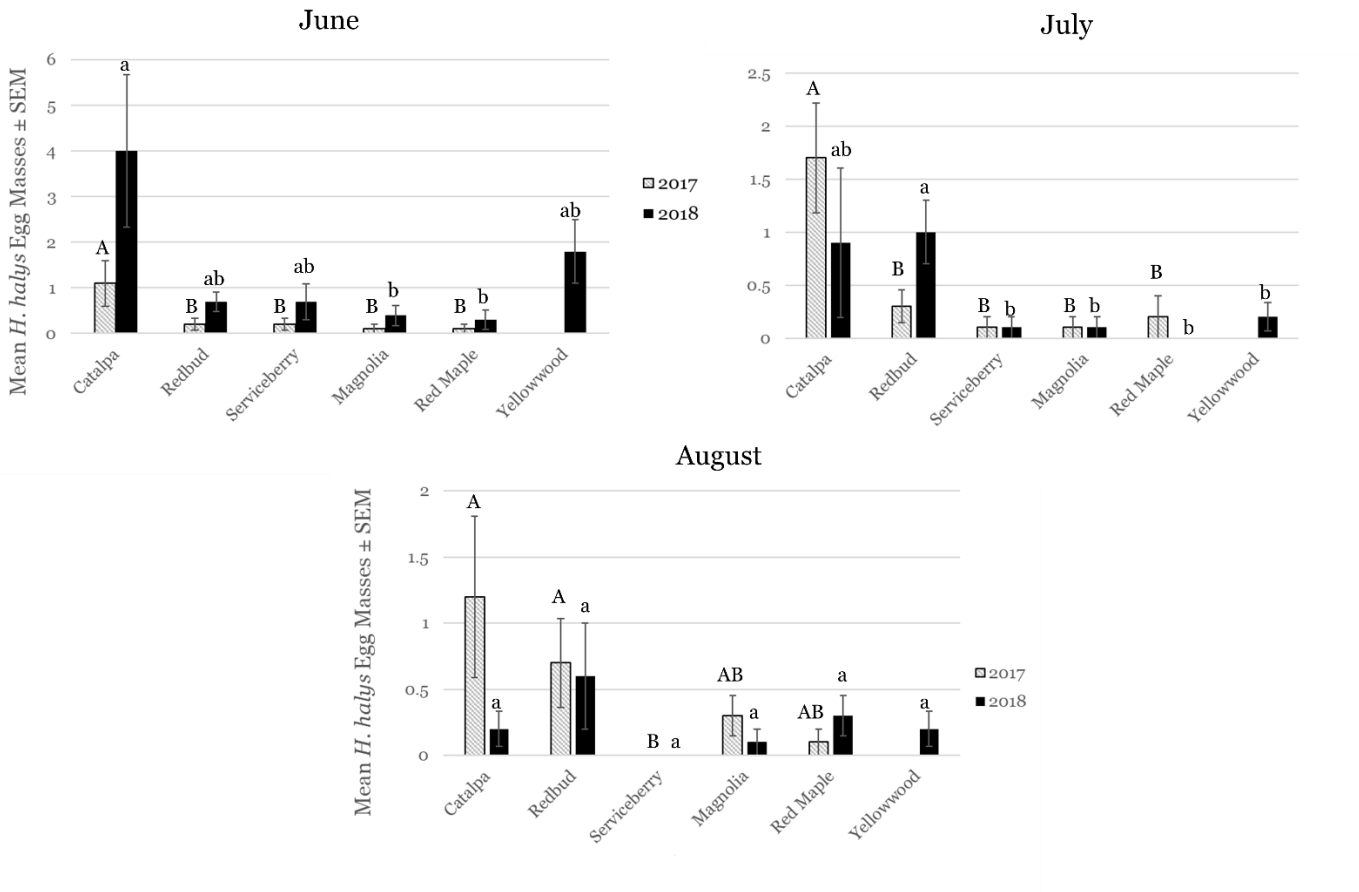


Figure 3.5. Mean (± SEM) number of *H. halys* egg masses collected during two-minute samples from ten trees per tree species. Letters compare number of egg masses found between tree species for each given year with uppercase letters showing 2017 results and lowercase showing 2018. Different letters report significantly different means at $P < 0.05$.

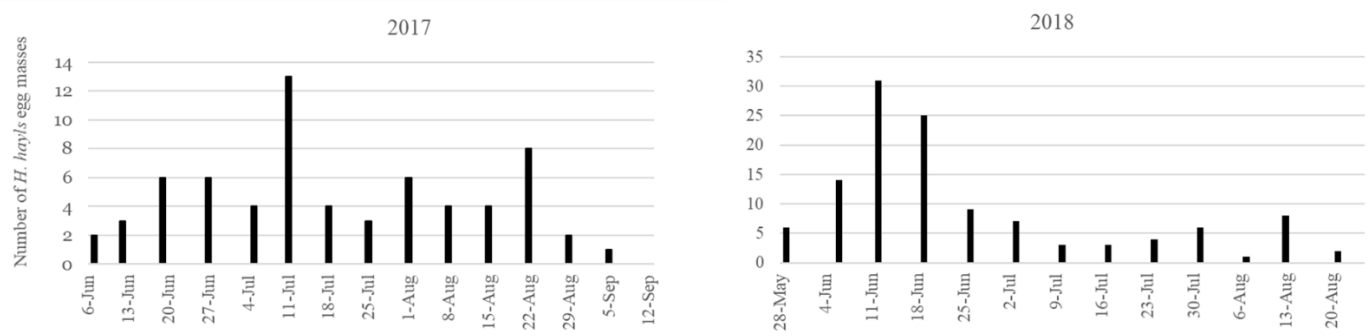


Figure 3.6. Total number of *H. halys* egg masses found each week on all tree species. Peak egg masses occur in early July in 2017, and early June in 2018.

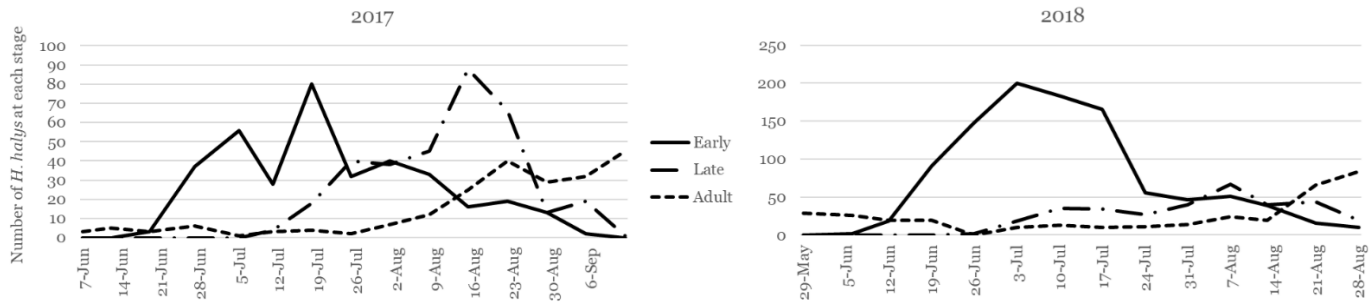


Figure 3.7. Life table trends. Early instars (2^{nd} & 3^{rd}), late instars (4^{th} & 5^{th}), and adult *H. halys* counts on all tree species throughout 2017 & 2018.

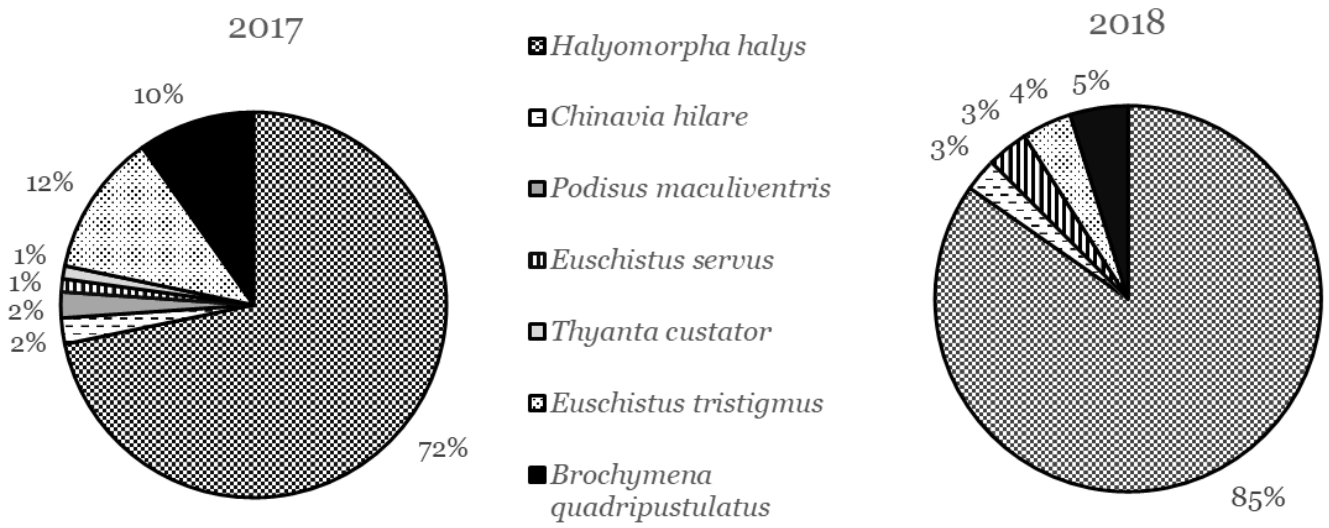


Figure 3.8. Percentage of stink bug egg masses found throughout the entirety of each field season on all tree species.

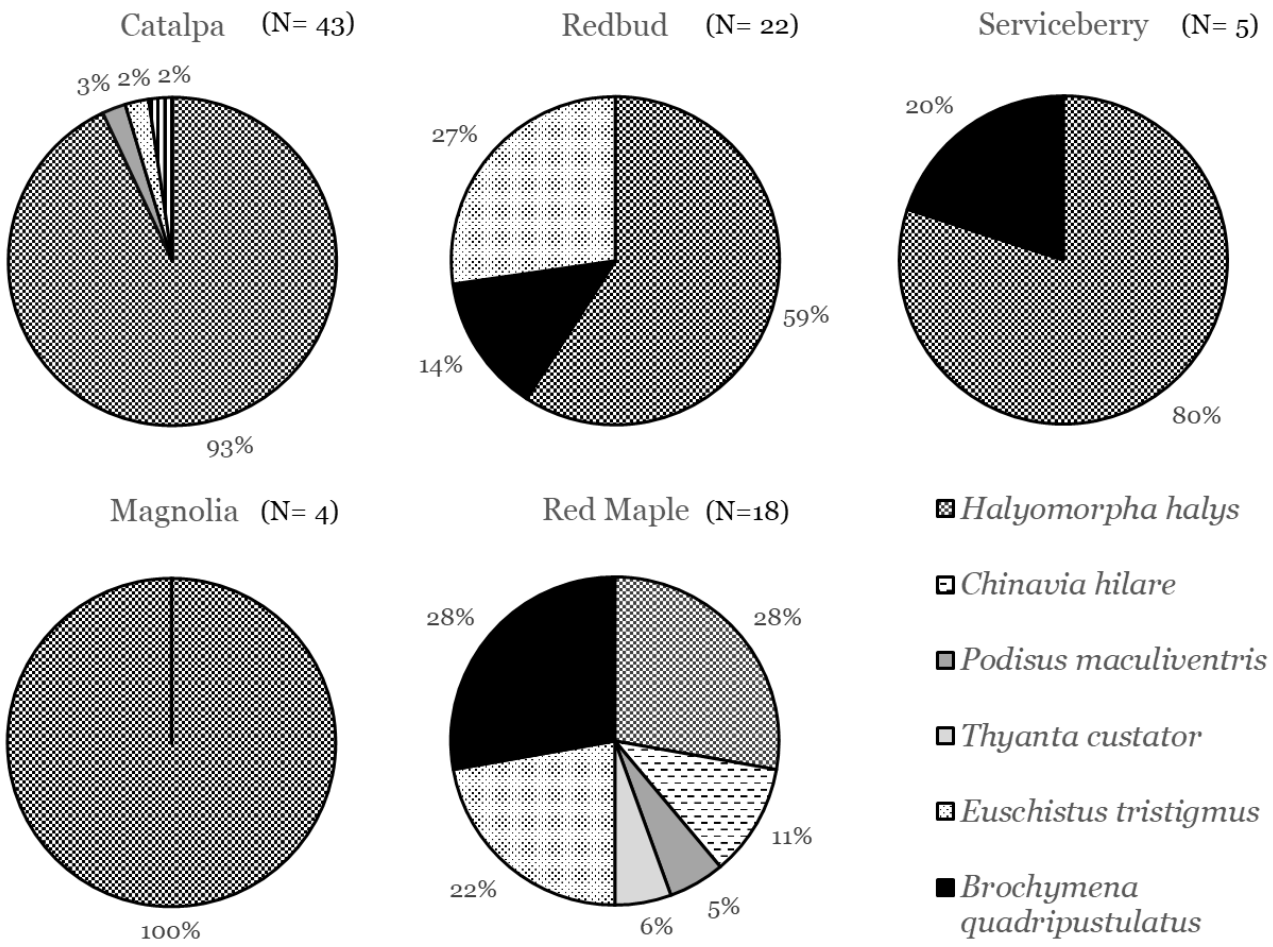


Figure 3.9. Stink bug species found in five tree species during 2017. Percentages show cumulative data from all samples and all trees in 2017. Total number of egg masses collected indicted near title as (N = #).

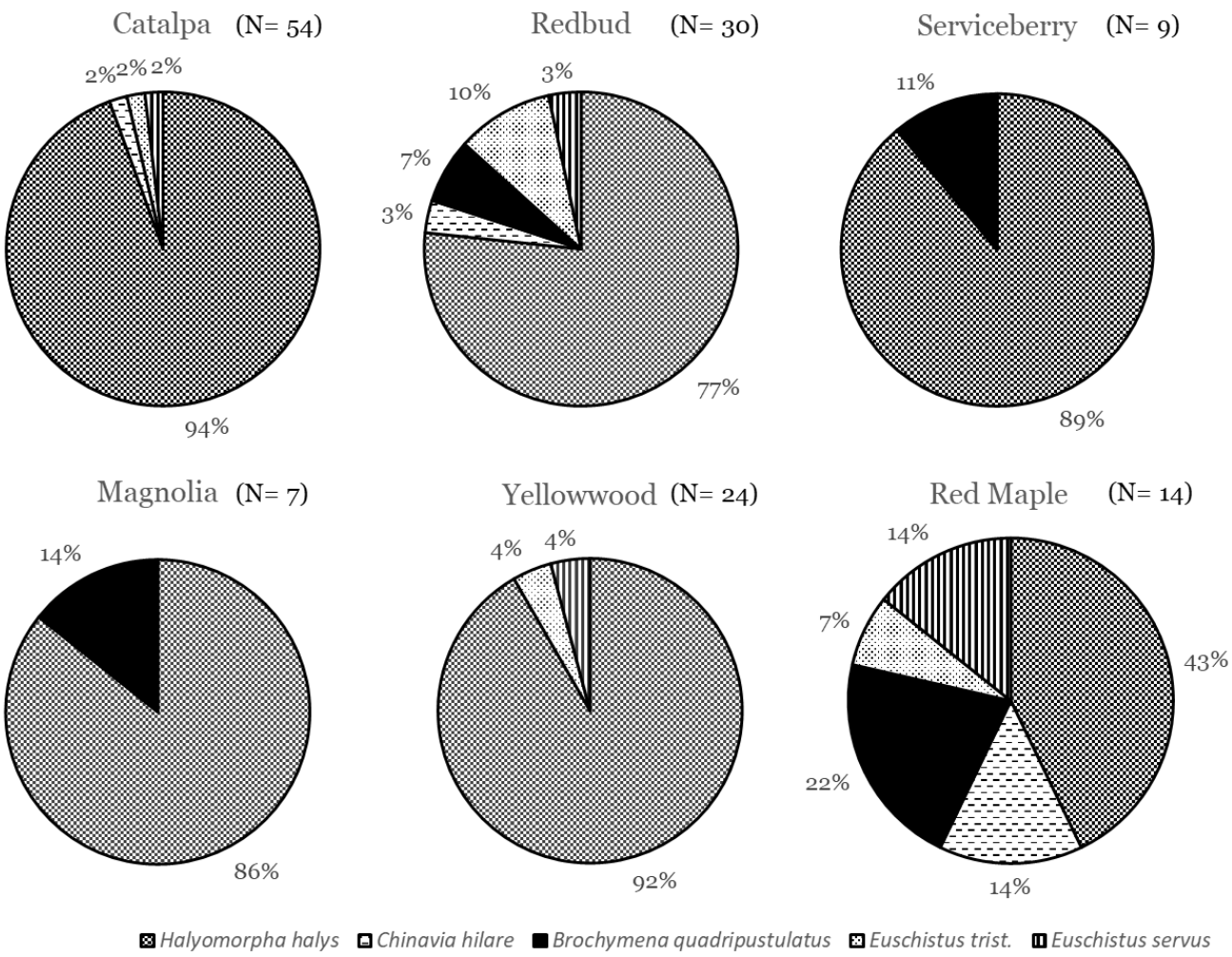


Figure 3.10. Stink bug species variation in six trees sampled in 2018. Percentages display cumulative counts from all sampling periods per tree species (N = total number of stink bug egg masses collected on each tree species).

Discussion

Among the trees sampled in our study, catalpa trees had the most egg masses throughout the summer, while redbud trees had similar numbers to catalpa in later summer months (Fig. 3.5). *H. halys* was also the most abundant stink bug found on catalpa trees at over 93% for both of the study years (Fig. 3.8 - 3.10). Therefore, catalpa trees may be an excellent candidate for augmentative releases of *T. japonicus* due to the high proportion of *H. halys* egg masses and the high numbers of eggs. Conversely, red maple may be a poor release site for *T. japonicus* for opposite reasons. Throughout the summers of both 2017 and 2018, red maple had the fewest *H. halys* egg masses and made up less than 50% of the stink bug egg masses found on the trees (Figs. 3.5, 3.8 - 3.10). As a result, if *T. japonicus* were to be released under these trees, the parasitoids would not have the optimal chance to parasitize *H. halys* eggs and may lead to non-target parasitism events due to the high numbers of native stink bugs found on these trees.

One factor that likely contributes to *H. halys* oviposition and tree selection is the presence of fruiting structures on the trees (Martinson et al. 2013, Leskey and Nielsen 2017). For example, serviceberry (or Juneberry) fruiting structures mature in early June and are shed by late summer (Ochterski 2011). Consequently, most of the *H. halys* egg masses deposited on this tree species were found in June, with declining numbers in July and no eggs found in August when fruit are scarce (Fig. 3.5). Conversely, redbud produces pod-like fruiting structures in late summer, matching when high numbers of *H. halys* egg masses were found on this tree species (Fig. 3.5). A final example of this trend was seen on yellowwood trees. In 2017, high numbers of *H. halys* egg masses were found on yellowwood when surveying for parasitoids and as a result, the tree species was added to the study to potentially compete with catalpa as the tree with the most numerous egg masses. However, in 2018, results showed that yellowwood was just marginally

better than tree species other than catalpa in the early summer and was one of the worst trees in the late summer (Fig. 3.5). This is most likely due to yellowwood being a biennial plant that produced pod-like fruit in 2017, but no fruit on the trees we sampled in Blacksburg in 2018 (Niemiera 2009).

Our study revealed no significant effect of *H. halys* aggregation pheromone lure on egg mass densities observed in trees in either of the sample years. These results were a little surprising as the pheromone lures described by Weber et al. (2014) have proven to be a strong attractant of *H. halys* adults and nymphs to baited traps and plants in the field (Leskey et al. 2015a,b, Weber et al. 2017). Thus, more *H. halys* adults gathering near the aggregation pheromone may not necessarily correlate with higher egg densities.

Surveying the collected egg masses resulted in no emergence of *T. japonicus* from parasitized eggs. Parasitism by native wasps was low for both years with rates of 11 % in 2017 and only 2 % in 2018. These rates are similar to rates reported on fresh *H. halys* eggs of 7% by Herlihy et al. (2015) and Dieckhoff et al. (2017). The differences in parasitism rates between years is most likely due to the timing of *H. halys* oviposition peaks. In 2017, peaks were later in the year, which allows wasps to complete a generation early in the year and their offspring can then parasitize eggs during the peak timing. In 2018, the peak happened early in the summer and relatively few egg masses were found after that point, resulting in a lower percentage of parasitized eggs. Still, these results mirror previous work that showed *Anastatus* spp. is the predominant native parasitoid of *H. halys* in woody habitats (Abram et al. 2017, Jones et al. 2017).

Ultimately, these results should be used to help make informed decisions about augmentative releases of *T. japonicus* in and around urban or suburban habitats where similar

ornamental tree species may predominate. Of the trees surveyed, catalpa had the highest number of *H. halys* egg masses throughout the summer, while having one of the highest percentages of *H. halys* eggs versus native stink bug egg masses, making it a prime candidate to release parasitoids near. For other localities, this study offers techniques to determine which of *H. halys'* vast host tree species are good candidates for release and offers insight on the relationship between the presence of fruiting structures and oviposition selection. All the while, these results demonstrate why native parasitoids are not enough to control *H. halys* populations and shows the need for another option for biological control.

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Chapter 4

Brown marmorated stink bugs with red pigmented abdomens: incidence, potential causal agent, and associated life span

Abstract

Halyomorpha halys (Stål), is an invasive stink bug from eastern Asia that has become a pest to a number of cultivated plants worldwide. Typically, adult *H. halys* have a mostly brown body with alternating black and white strips or "marmorations" on the antennae, fringe of the abdomen, and legs, with a white or tan sternum. Variations have been observed with red or pink sternal plates. Current understandings of this phenomenon are contradictory, some researchers believe it is associated with male bugs preparing to overwinter, while others hypothesize that it is the result of infection by a red bacterial pathogen, *Serratia marcescens*. In this study, we conducted laboratory assess to determine if 1) the red-sternum phenotype is more prominent in males, in agreement with those who hypothesis that this is a sexed biased overwintering phenotype and 2) the red phenotype is correlated with an increase in mortality, indicative of a potential bacterial pathogen, *Serratia*. Insects were collected in two years, 2017 and 2018 and observed for mortality. We found no difference in the proportion of red males to white males; however, there is a difference in the proportion of white females to red females. There was no difference in mortality across either sex or sternum color, and when dissected and cultured, one colony of *S. marcescens* was identified. These results suggest, in agreement with previous work, that red-sternum *H. halys* are likely preparing for overwintering.

Introduction

Natural populations of brown marmorated stink bug, *Halyomorpha halys* (Stål), have been observed with variations to their typical white sternum coloration. Variations include a range of pink to dark red hues, often with splotches of bright orange intertwined. This phenotypic variation in color was reported in Japan by Niva and Takeda (2002), who suggested that the red pigmentation was due to the presence of erythropterin. These authors hypothesized that the coloration was due to photoperiod and diapause, where adults that were exposed to diapause inducing conditions did not turn red and that when exposed to normal growth conditions, 16:8 (L:D) h, adults started to develop this red coloration (Niva and Takeda 2002). This phenomenon was further studied by the same authors, Niva and Takeda (2003) and it was determined that nymphal development also effected adult sternum coloration, where nymphs exposed to diapause inducing conditions turned into white-sternum adults, while under normal conditions had a higher proportion of red-sternum adults. It was also observed in both of these studies that males developed this coloration more rapidly than females and with a darker hue.

The red-colored abdomen has been frequently observed in U.S. populations of *H. halys* both in the wild and in laboratory colonies and has been a subject of curiosity among researchers of this invasive insect (*D. Weber* USDA-ARS, *T. Leskey* USDA-ARS and *T. P. Kuhar*, personal communication). Another potential source of the red sternum coloration in *H. halys* may be related to infection by the bacterium *Serratia marcescens* (Bizio), a gram negative rod in the order Enterobacteriales and family Enterobacteriaceae (Yu 1979). Bacterial colonies are red in appearance due to production of a red pigment, prodigiosin (Bennett and Bentley 2000; Khanafari et al. 2006). Pigment production varies depending on a number of different factors including incubation time and growth substrate (Giri et al. 2004). Due to this bright red pigment, *S.*

marcescens has been used throughout history, most notably in the 1950s where the U.S. government spread the bacteria in an attempt to study nuclear drift (Tansey 2004). Unfortunately, this event lead to multiple illnesses and even death, as *S. marcescens* was discovered to be a human pathogen (Kreston 2015; Sharanya et al. 2018). Similarly, *S. marcescens* has been shown to be an entomopathogen, killing a number of insects including *Helicoverpa armigera* and *Phyllophaga blanchardi* (Mohan et al. 2011, Pineda-Castellanos et al. 2015). One potential factor for *S. marcescens* entomopathogenicity is its production of extracellular chitinases. These enzymes have been reported at 10 times greater concentration in *S. marcescens* than in over 100 other bacterial species (Monreal and Reese 1969). It has also been shown that chitinases work synergistically with the red prodigiosin pigment to cause mortality and may be indicative of pathogenic strains (Someya et al. 2001).

Recently, Martin et al. (2016) filed a U. S. patent that described a strain of *S. marcescens* capable of killing *H. halys*. This bacterium was isolated from *H. halys* with dark red abdomens that were killed in less than 48 hours. This strain of *S. marcescens* was described as causing the abdomens of male *H. halys* to turn pink or red.

Herein, we report laboratory experiments to explore *H. halys* sternum coloration in Virginia populations. Populations of *H. halys* in western Virginia were studied to assess 1) if this red pigmentation is more prominent in males than females and 2) if this pigmentation is indicative of increased mortality. *H. halys* were dissected and the midgut and hindgut were plated to determine if *S. marcescens* were present in insects with red sternums.

Materials and Methods

Insects. Adult *H. halys* were collected from three sites in western Virginia in late-September 2017 and in early October 2018. In 2017, stink bugs were collected by hand at three locations: 1) the Jefferson National Forest shooting range in Montgomery Co., VA, 2) red maple trees in a development in Blacksburg, VA, and 3) from an overwintering cluster on the front porch of a home in Front Royal, VA. In 2018, one collection was made from the shooting range in Montgomery Co., VA. Insects were kept in 30.5-cm^3 screened cage (BioQuip Products Inc., Rancho Dominguez, CA) separated by location. Insects were counted and sex and coloration of their abdomen (red vs. white) was recorded. Insects were kept in cages at room temperature (~ 21 °C) and provided with water and an assortment of food including soybean, zucchini, tomatoes, grapes, carrots and peanuts.

Mortality Study. Adult *H. halys* were separated into four groups for each location: red male, red female, white male and white female. Insects were then placed individually into 30 mL soufflé cups with lids and provided with carrot as food. Soufflé cups were placed into development chambers set to 26 °C, 70% RH, and a photoperiod of 16:8 (L:D) h. Mortality was assessed every 24 h at the same time for two weeks to determine if abdomen color was indicative of shorter life span. During mortality assessments, cups with dead bugs were removed to reduce the spread of disease in the chambers. Food was replaced as needed.

Biochemical Identification. Dead *H. halys*, both red and white, from the mortality assay were dissected to observe their digestive tract. Five insects from of each of the four possible combinations of location and phenotype were randomly selected for biochemical testing to determine if *S. marcescens* was present in the alimentary canal. Mid and hindgut selects as well as gooey substances from the interior sternal plates were removed from the dissected bugs and placed

in 1 mL microcentrifuge tubes (Fisher Scientific International Inc., Hampton, NH) with 1 mL of 1 × PBS at a pH of 7.4 and were emulsified with forceps to suspend the bacteria. Large fragments of the digestive tract were then removed and the suspension was vortexed for > 5 seconds to mix the contents. The bacterial suspension was then diluted into 1 x PBS at a pH of 7.4 and followed a serial dilution scheme to 10^{-1} and 10^{-2} of the original concentration. Solutions were diluted using spread plate techniques onto LB agar to form 10^{-1}, 10^{-2}, and 10^{-3} dilutions of the original bacteria solution. Plates were incubated at 30 °C for 72 hrs. After incubation, plates were examined for colonies with similar morphology to *S. marcescens*. Putative colonies were removed and streaked onto LB agar and incubated for 48 hrs at 30°C. If these plates contained pure cultures, it was tested using API Rapid 20E kits for enteric bacteria (bioMérieux Marcy-l'Étoile, France), a biochemical test shown to successfully identify *S. marcescens* isolated from plants and other insects (Cooper et al. 2014). The API protocol for 20E strips was followed and identification was determined using the API system.

Data Analysis. All data were analyzed using JMP® Pro version 13 (SAS Institute Inc., Cary, NC, 2007). A two-way ANOVA was used to determine differences in sex and abdomen color in the populations of the stink bugs collected. Effects of sex and color were analyzed using Tukey post hoc test for multiple comparisons. Effects of sex, abdomen color, and the interaction between the two on mortality proportions were also compared using a two-way ANOVA. Level effects were tested using Tukey post hoc test and significance for all tests was determined at values alpha < 0.05.

Results

Two-way ANOVA for population dynamics showed no significant interaction effect, (t = -0.82, d.f. = 19, p = 0.4242) for effects of sex and abdomen coloration. Tukey post hoc test showed that there were higher proportions of white adults than red adult *H. halys* (p = 0.0207), with red females being significantly less than all other types (Fig. 4.1). These results are expected as the white sternum phenotype is the described phenotype of *H. halys* (Hoebeke and Carter 2003) and the population dynamics are similar to those described in Niva and Takeda (2002).

Mortality was low prior to the seven-day observation. The seven and 14-day observations were analyzed with two-way ANOVA, F (3, 8) = 0.0040, p = 0.9513, and F (3, 8) = 0.0013, p = 0.9725, and showed no significant interaction between sex and abdomen coloration. Taken individually, Tukey post hoc test showed no significant difference in mortality related to sex, or abdomen coloration for either time mark (Fig. 4.2).

Pink and red bacterial colonies were found from both white (N = 3) and red-sternum adults (N = 6) from the mid and hindgut sections; however, no bacterial colonies were produced from the substance found on the sternal plates. On these corresponding plates, pink and red colonies made up the majority of bacterial colonies. Only one plate had red colonies identified as *S. marcescens*. The bacterial colonies on this plate originated from a red-sternum adult and neither the adult stink bug nor the colonies were distinguishable from any of the other red adults or colonies. The oxidase test ruled most of the red/pink colonies out, as most colonies were oxidase positive, while *S. marcescens* is oxidase negative, proving to be an adequate screening step to improve efficiency of the test.

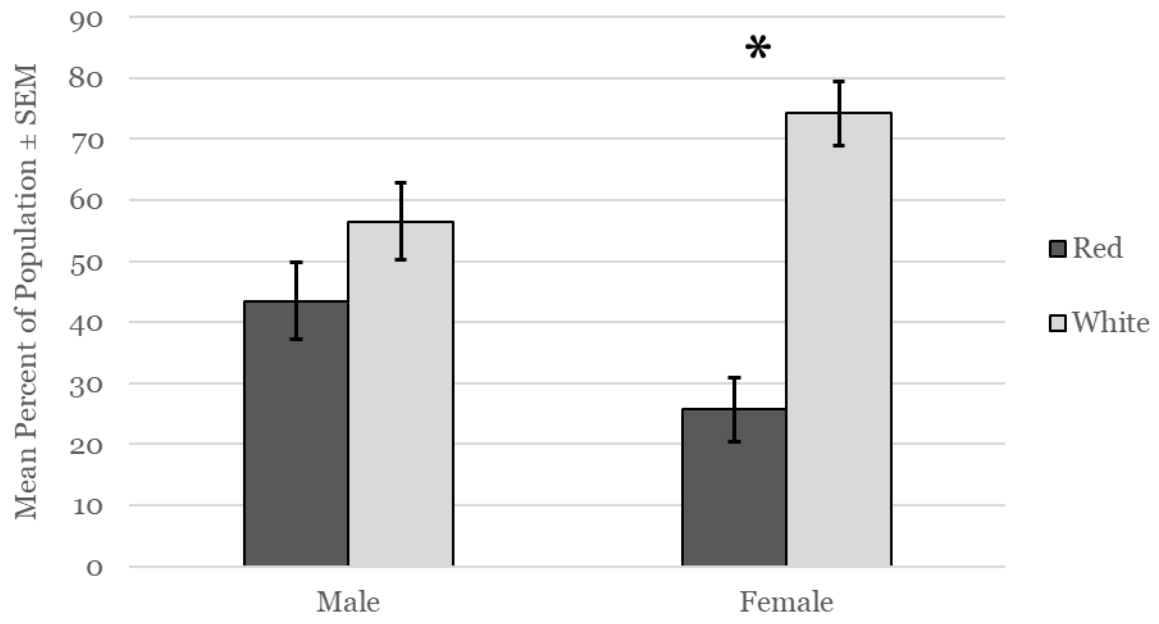

Figure 4.1. Percentages of *H. halys* adults with white versus red sternums based on collections from three populations in Virginia in 2017-18.

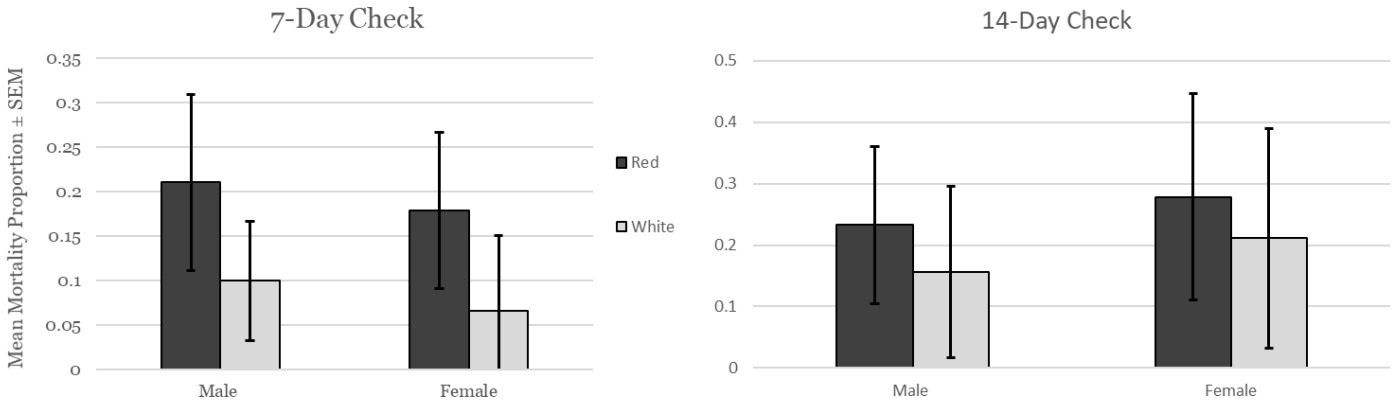

Figure 4.2. Mortality (mean ± SE) of *H. halys* adults grouped by sex. Dark bars indicate red-sternum adults, while light bars indicate white-sternum adults. Graphs show the seven day and 14-day time checks.

Discussion

Our results are similar to previous studies that show the red phenotype is more abundant in males than in females (Niva and Takeda 2002). In contrast, some populations of this red phenotype are not different in number to the white phenotype, suggesting that there should be an update to the species description described previously by Hoebeke and Carter (2003) to include this potential phenotypic variation.

Mortality was not significantly different between any of the groups at either of the time points checked (Fig. 4.2). Mortality was analyzed at seven and 14 days due to relatively low mortality until the 7-day point. If mortality were due to a bacterial infection of the entomopathogenic strain of *Serratia*, mortality is expected to be near 100% after two days (Martin et al. 2016). It is possible that different strains of *S. marcescens* elicit different levels of entomopathogenicity (Mohan et al. 2011). With that in mind, it cannot be concluded that red abdomen *H. halys* will behave similarly to white adults when used in mortality related bioassays and should be excluded when developing future experiments.

Red and white abdomen *H. halys* were dissected for biochemical tests. The digestive tract was often red coloration in both red and white *H. halys*. In the red adults, an unidentified red gooey substance was observed on the sternal plates. When this substance was transferred to LB agar, no bacterial growth occurred. Plated digestive tract solutions contained red bacterial colony growth from both red and white abdomen adults. In these red colonies, *S. marcescens* were ruled out with the oxidase test as *S. marcescens* is oxidase negative and the other red colonies were positive (Hejazi and Falkiner 1997). Only one of the red oxidase negative colonies was identified as *S. marcescens* with the API biochemical system. Another potential identity for the red bacteria could include species from the genus *Rugamonas*, a genus that also contains red oxidase positive bacteria

(Austin and Moss 1986, Whitman 2016). In future studies, oxidase tests may offer a more efficient screening process as it is an inexpensive, quick test that proved to be more important when determining if a colony might be *S. marcescens*. Using color alone was not an effective screening tool, as not all red colonies are *S. marcescens* and the bacteria does not always produce red pigmented colonies (Flyg et al. 1980).

In this study, we explored mortality in *H. halys's* red phenotype and tested for *S. marcescens* bacteria. The results show that this red abdomen is more common in males than females. This red coloration had no effect on mortality at either seven or 14 days and was likely not the result of *S. marcescens*. Interestingly, the red coloration that was plated from the interior of the sternum did not lead to bacterial growth. Potentially, the red substance found on the *H. halys* sternum is erythropterin, the substance identified by Niva and Takeda (2002). Future studies could determine if this red substance is erythropterin with similar methods to the Niva and Takeda study from 2002 using chromatography.

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Chapter 5

Summary and Conclusions

Information regarding *H. halys* ecology was researched under laboratory and field settings. Four experiments were conducted including: development on different hosts, oviposition on tree hosts, the occurrence of egg parasitoids and other biological control agents. Results from these experiments can help provide a foundation for future management tactics. This section includes pitfalls we encountered and suggested improvements to the methodology listed in previous chapters, as well as ideas for future research and how this research may be applied to control.

Improvements to methodology

There were a number of challenges with the methodology of the survival and development on vegetable host study. It was difficult to maintain potted plants in small screen cages. The best outcome was achieved by planting new transplants each week of the study. For the course of the study, replacing the plants frequently provided optimal materials for the nymphs. Similarly, it was difficult to keep the fruiting structures free of mold for prolonged periods. Once mold formed in a development cage, it became necessary to replace the fruit to reduce the spread of mold and diseases in the cage. The final and most difficult challenge was locating all of the nymphs, both dead and alive, in the development chambers. This was particularly difficult with small, agile second and third instars, who would often move, disrupting counts. To combat this, survival counts were done multiple times per sample period and only on live nymphs. Live counts proved more reliable than counting dead nymphs since exuviae often look similar to dead nymphs and could lead to conflicting results.

Challenges also arose during the tree host study. One key issue was tree host health. Throughout the course of the two-year study, several trees became sick and lost their leaves during the summer. This was an issue because *H. halys* lays the majority of its eggs on the leaves. To combat this, surveyed tress were replaced as necessary with healthy trees of the same species and phenology. At the start of the second year, we planned to use the same trees as the first year; however, there were multiple trees that had been removed, as the trees were situated in an urban setting. When necessary, we replaced the surveyed tree with a nearby healthy tree of the same species and general size. The final challenge of this study was dealing with counting large numbers of nymphs throughout the season when surveying *H. halys* nymphs in trees. It was not uncommon to find over 50 nymphs in a given tree, and trying to count, determine the instar, and find egg masses was often difficult. To combat this, we performed multiple searches where during the first search only egg masses were be counted, and then in the second search only nymphs were counted. If egg masses were found in the second search, they were not counted and left in the tree and similarly, if nymphs moved from the tree after the first search, they were not counted in the second search.

In the chapter relating the red-sternum to *S. marcescens*, collecting enough stink bugs to have 20 red females was often difficult. To make this manageable, adult bugs were collected at overwintering sites in mass numbers. Biochemical tests proved to be time consuming and used a large quantity of materials. One way to make this process more time and cost efficient is to use the oxidase test as a primary screening test for *S. marcescens*. Few of the colonies isolated from *H. halys* digestive tracts were oxidase negative like *S. marcescens*, allowing us to use this step in the biochemical test process to reject some of the unknown colonies.

Applications and Future Directions

Results from these studies can help us shape *H. halys* management plans in the future. For example, the results from the vegetable host study show which vegetables *H. halys* complete development on with the highest survivability. This can also explain why some vegetables, like sweet corn, have been observed with high populations during the growing season. This information can be used by farmers who live in areas with high populations of *H. halys*, as they can determine which crops to scout first for *H. halys* populations. In the future, this study could be expanded upon to increase the number of vegetables tested and potentially add more vegetables that produce defensive compounds. It would also be interesting to see if these results can be confirmed in the field.

The study with the most promise for immediate application is the ovipositional study looking at tree hosts *of H. halys* around Blacksburg, Virginia. The methods in this study should be applied to areas other than southwestern Virginia to determine which trees *H. halys* lays the most egg masses on. This will be important knowledge for the future releases of *T. japonicus* and other potential biological control agents that target *H. halys* egg masses. In the future, this study could be improved upon by adding more tree species as well as looking at tree species in different types of environments, such as wooded farm borders and other forested environments. Another limitation of our study was that the experiment was not set up to take location into consideration. Future studies should do so by trying to find all tree species at one location to determine if locational differences have an effect on the number of egg masses found.

The last study that has practical application is the determination of the red-sternum phenotype. This study showed that this phenotype was not responsible for higher mortality than the normal white phenotype from the populations that we collected. We were also not able to

identify *S. marcescens* as the causative agent for this phenotype in our collections. Perhaps red pigment is associated with bugs preparing for overwintering. In the future, more work needs can be done to determine why some of these stink bugs produce this pigment and if there is an explanation as to why it is more prevalent in males than females.