LIFE HISTORY INVESTIGATIONS OF HEMIPTERANS SELECTED FOR NON-TARGET HOST-SUITABILITY STUDIES OF THE SPOTTED LANTERNFLY (*LYCORMA DELICATULA*) BIOCONTROL AGENT *ANASTATUS ORIENTALIS*, WITH MOLECULAR INVESTIGATIONS OF LOCAL *ANASTATUS* TAXA

by

Tyler Hagerty

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ABSTRACT

This project consisted of two separate objectives. First, the phylogenetics of the genus *Anastatus* (Hymenoptera: Eupelmidae) north of Mexico was investigated by extracting and amplifying the mitochondrial cytochrome c oxidase subunit 1 (CO1) to use as a comparison and identification tool. This genus was the focus due to the genus containing species that have attacked spotted lanternfly (*Lycorma delicatula*) eggs in Asia. Five species of *Anastatus* were extracted but only *A. reduvii* was successfully amplified. This resulted in no phylogenetic investigation into the genus. The second objective was to investigate the life histories of native hemipteran species and establish rearing colonies to acquire eggs and nymphs from said species. Those eggs and nymphs were collected to provide material for cooperating labs conducting non-target host suitability testing for the biological control of the spotted lanternfly (*Lycorma delicatula*). Fourteen species in total were studied during the course of this research and eggs were obtained from eight of them (*Poblicia fuliginosa* (Fulgoridae), *Rhynchomitra microrhina* (Dictyopharidae), *Flatormenis proxima* (Flatidae), *Acanalonia conica*, *Acanalonia bivittata* (Acanaloniidae), *Thelia bimaculata* (Membracidae), *Arilus cristatus* (Reduviidae), *Anasa tristis* (Coreidae)).Nymphs were obtained from six (*Poblicia fuliginosa* (Fulgoridae), *Rhynchomitra microrhina* (Dictyopharidae), *Flatormenis proxima* (Flatidae), *Acanalonia conica*, *Acanalonia bivittata* (Acanaloniidae), *Anasa tristis* (Coreidae)).

Chapter 1

THE ECOLOGY AND LIFE HISTORY OF THE INVASIVE SPOTTED LANTERNFLY (*LYCORMA DELICATULA*)

The spotted lanternfly (SLF), *Lycorma delicatula* (White) (Hemiptera: Auchenorrhyncha: Fulgoromorpha: Fulgoridae) (Fig. 1), is an invasive phloem feeding insect originally described from China in 1845 (White 1845, Urban 2019). In the US, it was initially reported in Berks County, Pennsylvania on September 22, 2014, where it had evidently been introduced 2-3 years earlier (Barringer et al. 2015).

Lycorma delicatula (SLF thereafter) is a univoltine hemipteran with four instars (most Auchenorrhyncha have five). The first three instars have black bodies with a characteristic pattern of white spots. The fourth instar is predominately red, with black markings and distinct white spots. This transition from black to red aposematic coloration corresponds to a transition in nymphal feeding habits from polyphagy to a strong preference for tree of heaven (*Ailanthus altissima* (Mill.) Swingle). This aposematic coloration continues into adulthood. Adults measure17-25 mm long with greyish forewings and red and black underwings (Barringer et al. 2015). The strong preference towards tree of heaven is where the insect evidently acquires and sequesters compounds presumed to be distasteful and which may provide defense against predators. This interaction is speculative, and studies are currently being conducted focusing on the interaction. The main compound is ailanthone, a quassinoid acquired from *Ailanthus* (Song et al. 2018). Eggs are deposited in groups of 30-50 on vertical surfaces such as tree trunks, branches and telephone poles (but also sometimes

on rocks and man-made objects), and are covered in a waxy coating which hardens, forming an oothecum (Dara et al. 2015) (Fig. 2). It appears that SLF was initially transported to the US as eggs on stone products, apparently from China (Barringer et al. 2015). Similar egg cases have been reported for other species of Fulgoridae (O'Brien 1988, Hogue et al. 1989).

SLF is associated with over 70 plant species including fruit trees, ornamental trees, vines and woody trees (Park et al. 2009, Dara et al. 2015). Beginning with the fourth instar, it exhibits a notable preference for tree of heaven, but has been shown to develop on several other plant species (Liu 2019). This vast selection of plant associations makes it difficult to target for control. Besides *Ailanthus*, grapes (*Vitis spp.*) are also a favored host, which could make SLF an important pest in vineyards for both juice grapes and viticulture (Dara et al. 2015). In September of 2017 the spotted lanternfly was found in large numbers in a fruit orchards including apple (*Malus sp.*), peach (*Prunus persica* (L.) Batsch) and nectarines (*Prunus persica* var. *nucipersica* (Suckoq) C.K. Schneid.,), which greatly increases its potential as a pest species. They consume and excrete large amounts of fluids, which coat all parts of the plant below them (stems, leaves, trunks). Their sugar-rich excretions hasten the growth of sooty mold, which can weaken, and possibly kill, plants when combined with heavy feeding (Kim, Lee, et al. 2011, Urban 2019). The history of the SLF introduction into Korea in 2006 shows that the species has the capacity to spread rapidly if not controlled (Han et al. 2008, Park 2015).

SLF has spread since the original introduction to Berks County despite vigorous efforts by the Pennsylvania Department of Agriculture (PDA) to control the species (Urban 2019). PDA quarantined SLF to limit the movement to new locations

and to aid in eradication efforts. The quarantine requires those located or working within the area to obtain a permit to move vehicles, products or other conveyances within or out of the spotted lanternfly quarantine zone (PDA 2018). The spotted lanternfly quarantine now includes 23 counties in Pennsylvania (Berks, Bucks, Carbon, Chester, Columbia, Cumberland, Dauphin, Delaware, Huntington, Juniata, Lancaster, Lebanon, Lehigh, Luzerne, Mifflin, Monroe, Montgomery, Northampton, Northumberland, Perry, Philadelphia, Schuylkill, York) (Cornell University 2019).

In addition to Pennsylvania, reproducing SLF populations are now reported in Delaware, New Jersey, Maryland, Virginia and West Virginia. Individuals of SLF have also been reported in New York, Connecticut, North Carolina and Massachusetts. New Jersey placed three counties (Warren, Hunterdon and Mercer) in quarantine in 2018, and has since expanded its quarantine to include Somerset, Burlington, Camden, Gloucester and Salem counties. SLF has also been reported from Cape May County New Jersey (Cornell University 2019). Virginia originally reported detection of the spotted lanternfly in Winchester in January 2018. Since then Virginia has placed Frederick County and the city of Winchester into quarantine as of May 2019 (Lidholm 2019), and this population has since spread into adjacent Clarke County., Virginia and Berkeley Co., West Virginia. In February of 2019 Delaware placed a quarantine on several zip codes in New Castle County, but in September 2019 it was expanded to include all of New Castle County north of the Chesapeake and Delaware Canal (Delaware Department of Agriculture 2019), and populations have been found in adjacent eastern Cecil and northeastern Harford Counties, Maryland. The lanternfly has also been observed in Kent County, Delaware, outside of the quarantine zone (Delaware Department of Agriculture 2018). In New York, individual adult spotted

lanternflies has been observed in Erie, Monroe, Ontario, Yates, Chemung, Broome, Delaware, Albany, Westchester, Kings and Suffolk counties (Cornell University 2019). Hartford County Connecticut and Suffolk County Massachusetts have also reported observations (Cornell University 2019). Isolated adult sightings are presumed to be hitching insects moving with vehicles. With the rapid spread of the spotted lanternfly, several control and eradication methods are being tested.

There are many techniques used to control invasive insects, most notably traps and pesticides. For SLF, sticky bands have been used for monitoring and control, following and modified from methods initially used in South Korea (Choi et al. 2012, Dara et al. 2015). This trap is a simple sticky band, such as a premade trap for insects or duct tape placed with sticky side out, wrapped around a tree. The trap is effective at catching SLF nymphs, but bycatch (non-target organisms getting caught in the trap) can be a problem. Reported bycatch includes small mammals, birds, reptiles and many non-target insects (Etters and Leach 2019). Trap trees have also been used to help control SLF numbers on properties containing *Ailanthus*. Trap trees involve clearing a large amount of *Ailanthus* from a location and leaving a small number of male trees still standing. Those trees are then treated with a systemic insecticide (PDA has elected Dinotefuran bark sprays). These few trees then attract SLF which results in mortality (PDA 2019). Traps using chemical lures to limit bycatch are currently being researched. SLF evidently does not utilize pheromones (pheromones are rarely reported among Auchenorrhyncha (Nault et al. 1974, Chen and Liang 2015, Bartlett et al. 2018)), but plant volatiles may prove to be an effective lure. Three kairomones have been identified from *Ailanthus altissima* and *Vitis vinifera* (L.) that have been shown to be attractive to spotted lanternfly, methyl salicylate, (*Z*)-3-hexenol, and

(*E,E*)-α-farnesene (Cooperband et al. 2019). However, a successful lure is not yet commercially available. Several pesticides targeting eggs, nymphs, and adults are currently being researched for efficacy. Some of these chemicals include chlorpyrifos which has shown to be a viable insecticide on eggs (Kim et al. 2010, Shin et al. 2010). Two coepizootic fungal pathogens, *Batkoa major* ((Thaxter) Humber) and *Beauveria bassiana* ((Bals.-Criv.) Vuill.), have also been observed to be lethal to SLF in the United States (Clifton et al. 2019).

Biological control is currently being investigated as a longer-term control method for spotted lanternfly control (Lee et al. 2019, Urban 2019). Biological control consists of living, natural enemies targeting pest life histories and reducing their populations (e.g., Flint & Dreistadt, 1998).

Table 1. List of biological control agents for spotted lanternfly (all Hymenoptera)

Parasitoid Species	**Family**	**Type of Parasitoid**	**Reference**
Dryinus browni (Ashmead) /Dryinus sinicus* **(Olmi)**	Dryinidae	Nymph	Mita 2009, Kim et al. 2010, Dara et al. 2015, Urban 2019
Ooencyrtus kuvanae **(Howard)**	Encyrtidae	Egg	Liu and Mottern 2017, Liu 2019, Urban 2019
Anastatus orientalis **(Yang)**	Eupelmidae	Egg	Kim, Koh, et al. 2011, Choi et al. 2014, Dara et al. 2015

**Dryinus browni* currently thought to be *D. sinicus* (Xin et al. 2020)

Efforts to find a biological control agent for SLF have discovered three potentially effective hymenopterous natural enemy species (Table 1). The species

Dryinus sinicus (Olmi) (Dryinidae: Hymenoptera) has been discovered to parasitize nymphs in South Korea (Mita 2009) and China (Xin et al. 2020). *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae), an Asian species introduced early in the 20th century for gypsy moth control, has been reported to parasitize SLF eggs at a few field sites in Pennsylvania (Liu and Mottern 2017). An egg parasitoid, *Anastatus orientalis* (Hymenoptera: Eupelmidae), has also been observed parasitizing SLF egg masses (Kim, Koh, et al. 2011, Choi et al. 2014, Yang et al. 2015). *Anastatus* appears to be a promising control agent with higher parasitism rates and the ability to kill the eggs before hatching (Dara et al. 2015). In the native range of SLF in China, egg parasitism rates are as high as 32.98% in Haidian district, Beijing, and 68.96% in Yantai, Shandong Province. A small number of egg masses from Haidian were 100% parasitized and 23.6% of egg masses from Haidian were parasitized at ~50% (Choi et al. 2014).

There are 19 reported species of *Anastatus* in North America, out of approximately 154 species worldwide (Burks 1967, Krombein 1979, Noyes 2019). The biology and taxonomy of the genus are discussed and a generic key given (Burks 1967) based solely on the features of the females of each species. Males generally cannot be identified except through association with females by rearing (Burks 1967). Host associations (i.e., species parasitized) are mainly determined through rearing of wasps from parasitized eggs. Reported hosts for *Anastatus* include species from the orders Blattodea, Hemiptera, Hymenoptera, Lepidoptera, Mantodea, Neuroptera, Orthoptera and Phasmatodea (Gibson 1995).

Due to the difficulty of identification based solely on morphological features for the genus, molecular tools are being utilized to shed some light on cryptic species as well as provide a sound method for species identification. DNA barcoding is a promising way to help delineate species identification (Hebert and Gregory 2005). The cytochrome c oxidase subunit 1 mitochondrial subunit (CO1) has been used frequently for species discovery and identification (Folmer et al. 1994, Smith et al. 2006), and could prove to be a useful tool to increase the understanding of the genus *Anastatus*.

Both augmentation (mass releases to raise population levels of already occurring control agents) or introduction of novel species for biological control can have impacts on non-target native species. It is necessary to investigate impacts of biological control of non-targets to insure that detrimental effects on the ecosystem are avoided (NAPPO 2015). Most plausible non-target species for the release of *Anastatus* for the control of SLF would include (but are not limited to) those species phylogenetically closely related to SLF (larger planthoppers, mainly fulgoroids), with large body size and similar biology. However, phylogenetically more distant taxa, such as within the Cicadomorphs (leafhoppers, treehoppers, spittlebugs and allies) and Hemiptera (true bugs) may also be impacted. This is plausible given that *Anastatus* species are being investigated for control of stink bugs (Pentatomidae) (Dieckhoff et al. 2017, Stahl, Babendreier, et al. 2019)

The purpose of the research presented here has two parts. First, to investigate the systematics of local *Anastatus* species that might be useful as hemipteran biological control agents, and which might also attack SLF in the United States. Second, to investigate hemipteran species that may serve as subjects for host

suitability studies for potential SLF biological control agents. The objectives are 1) to sequence CO1 barcode gene data from available local species of *Anastatus* to test the current morphology-based taxonomy of *Anastatus*, and 2) to field collect and rear hemipteran species to produce eggs (and nymphs) for host suitability studies. As part of the second objective life history events will be observed and rearing techniques will be developed based on those observations.

Figure 1. Adult spotted lanternfly (*Lycorma delicatula*)

Figure 2. Covered *Lycorma delicatula* egg mass

Chapter 2

GENETIC ANALYSIS OF THE GENUS *ANASTATUS* (HYMENOPTERA: EUPELMIDAE)

2.1 Literature Review for *Anastatus*

The genus *Anastatus* currently contains 154 species described worldwide (Noyes 2019). There are 19 species reported in the United States. A key was made by Burks in 1967 to identify 13 of those species (Burks 1967); however, sexual dimorphism, and underrepresentation of males within collections, makes morphology-based keys only useful for females. Males can only be identified to species if they are kept in a rearing colony where they can be associated with females (Burks 1967). All native species of *Anastatus* in North America are primary egg parasitoid of several orders of insects, making them versatile biological control agents. *Anastatus tenuipes* (Bolivary Pieltain) is a successful parasitoid of *Supella supellectilem* (Seville) (brown-banded roach) and even traveled with its host into the United States (Roth and Willis 1960).

In 2011, the discovery of an egg parasitoid attacking invasive populations of SLF in South Korea was reported, based on collections of egg masses from *Vitis vinifera* in Cheongwon-gun, (Chungcheongbuk-do province), South Korea, on April 16th, 2010 (Kim et al. 2011). Egg masses were kept under observation in rearing sleeves until parasitoid emergence. The wasps were later identified to the genus *Anastatus* and this was the first record of an egg parasitoid of SLF. An attempt to

identify the wasps to species was conducted but no species identification could not be confirmed.

In 2015 a new species of *Anastatus* was described from SLF eggs based on its morphological characteristics (Yang et al. 2015). Overwintering eggs of SLF were collected from five regions in China (Yanglin, Shaanxi Province; Qinhuangdao, Hebei Province; Yantai, Shandong Province; Guangang, Tianjin; Haidian, Beijing). The entirety of the egg mass as well as the bark they were attached to were removed, placed in glass vials and kept at room temperature (20-25˚C). The parasitoid wasps that emerged from the collected egg masses were preserved in alcohol before being dried and mounted for study. Upon morphological study of the wasps, it was determined that the species was in the subgenus *Anastatus* (*Anastatus*) and was close to both *A. japonicus* (Ashmead) and *A. bifasciatus* (Geoffroy) but did not fit either species. Therefore, it was described as a new species, *Anastatus orientalis*. Later it was determined that the egg parasitoid described in the 2011 study by Kim et al. was also *A. orientalis*. During the study, a total of 450 egg masses from SLF were collected, and 137 of them were parasitized by *A. orientalis*. The parasitism rate of egg masses was 30.4% and the sex ratio of female to male was 1.9:1.0. The individual egg parasitism rate was 40.2%. Parasitized eggs can be distinguished from non-parasitized eggs based on the shape of the emergence holes, with the former having a round hole and the latter a long elliptical hole with the egg lid attached to the egg shell (Yang et al. 2015).

This discovery of egg parasitoids of SLF increased the need for a reliable method for species identification within the genus *Anastatus*. Because of the difficulties in identifying species within this genus due to the subjective nature of the

morphological characters (sculpturing of scutellum being either very finely sculptured or coarsely punctate), genetic markers were explored to help confirm species identity.

Barcode of Life (BOLD) currently has 137 public records of *Anastatus* representing 38 species from 19 countries. Only five of the records are collected from the United States. NCBI has 75 records for *Anastatus* representing nine species. GenBank has 26 records for *Anastatus* with 20 of them having a species identification.

Molecular analysis for species identification often utilizes different specific genes depending on the taxonomic group. One common gene used to resolve taxonomic identity is the mitochondrial cytochrome c oxidase subunit 1 gene (CO1) due to its conservative protein-coding regions (Hebert and Gregory 2005, Smith et al. 2006). A "universal" primer set for this gene was created by Folmer et al. (1994) and tested on 11 invertebrate phyla (or collective groups) consisting of Echinodermata, Mollusca, Annelida, Pogonophora, Arthropoda, Nemertinea, Echiura, Sipuncula, Platyhelminthes, Tardigrada, and Coelenterata. The primers created were LCO1490 (5'-ggtcaacaaatcataaagatattgg-3') and HC02198 (5'-taaacttcagggtgaccaaaaaatca-3') and they consistently created a 710-bp fragment of the CO1 gene (Folmer et al. 1994). These primers have been used to discern the presence of cryptic species within many families, including Hymenoptera, and have also been used to discover cryptic species in parasitoids (Hernández-López et al. 2012).

A recent study conducted by Stahl et al. designed primers to detect unemerged *Anastatus bifasciatus* within *Halyomorpha halys* (Stål) eggs. The primers Ana-361F (5'- atcacataggggtccttcagta-3') and HCO2198 (5'-taaacttcagggtgaccaaaaaatca-3') were used. They amplified a 320-bp fragment of CO1 from three different species of *Anastatus* (*A. bifasciatus, A. japonicus, Anastatus sp.*)(Stahl, Gariepy, et al. 2019).

The objective of this research was to morphologically identify *Anastatus* species occurring in the eastern United States north of Mexico. Those specimens identified morphologically were then processed for molecular characterization by extracting the targeted CO1 gene. Extracted sequences were then phylogenetically analyzed using Bayesian Inference (BI) and Maximum Parsimony (MP). Genetic analysis was used to help understand and recognize the morphological variation that could be used for species identification as well as recognize cryptic species if they occur. This was done because it is plausible that a native species of *Anastatus* could be discovered as a biocontrol agent for SLF.

2.2 Methods

All extractions were done using a DNeasy Blood & Tissue Kit (Qiagen, Toronto, ON) with methods listed in (Appendix A). Primers were purchased through ThermoFisher custom oligo creator (Waltham, MA). Primers were made into a 200µmolar stock and then diluted based on requirements needed. PCR purifications were done using a PCR purification kit (Qiagen, Toronto, ON) with methods listed in Appendix D. Sanger Sequencing was conducted at the Delaware Biotechnology Institute (Newark, DE) on an Applied Biosystems Sanger sequencer (ThermoFisher).

Taq DNA Polymerase recombinant for PCR amplification was purchased from ThermoFisher (Invitrogen, Carlsbad CA). Other PCR recipe components include Invitrogen 10X PCR Rxn Buffer (-$MgCl_2$), Qiagen 25 mM $MgCl_2$, Invitrogen 50 mM $MgCl_2$, and Invitrogen 10 mM dNTP Mix, all purchased from ThermoFisher.

Several different combinations of PCR recipes, primers, and thermocycle steps were used for each species and are numbered below.

PCR Methods

1) A 50µl PCR reaction consisting of 6µl of genomic DNA, 5µl of 10xPCR Buffer (15mmol/µl $MgCl_2$), 3µl of 25 mmol/µl $MgCl_2$, 1µl of 10 mmol/µl dNTP's, 1µl of 10µmolar HCO2098, 1µl of 10µmolar LCO1490, 0.5µl of Taq, and 32.5µl of nuclease-free H_2O. Reactions were amplified using the following parameters: one cycle at 94˚C for 2 minutes followed by 35 cycles of 94˚C for 30 seconds, 45˚C for 30 seconds, 72˚C for 2 minutes, and a final extension at 72˚C for 5 minutes.
2) A 25µl PCR reaction consisting of 1.0µl of genomic DNA, 2.5µl of 10xPCR Buffer (15mmol/µl $MgCl_2$), 1.25µl of 25 mmol/µl $MgCl_2$, 0.125µl of 10 mmol/µl dNTP's, 0.25µl of 10µmolar LepF1 primer, 0.25µl of 10µmolar LepR1 primer, 0.125µl of Taq, and 19.5µl of nuclease-free H_2O. Reactions were amplified using the following parameters: one cycle at 94˚C for 1 minute followed by 35 cycles of 94˚C for 40 seconds, 58˚C for 40 seconds, 72˚C for 1 minute, and a final extension at 72˚C for 5 minutes.
3) A 25µl PCR reaction consisting of 1.0µl of genomic DNA, 2.5µl of 10xPCR Buffer (15mmol/µl $MgCl_2$), 1.25µl of 25 mmol/µl $MgCl_2$, 0.125µl of 10 mmol/µl dNTP's, 0.25µl of 10µmolar LepF1 primer, 0.25µl of 10µmolar LepR1 primer, 0.125µl of Taq, and 19.5µl of nuclease-free H_2O. Reactions were amplified using the following parameters: 1 minute at 53˚C, 35 cycles of 10 seconds at 95˚C, 30 seconds at 55˚C, 1 minutes at 72˚C, with a final extension of five minutes at 72˚C.
4) A 25µl PCR reaction consisting of 1.0µl of genomic DNA, 2.5µl of 10xPCR Buffer (15mmol/µl $MgCl_2$), 1.25µl of 25 mmol/µl $MgCl_2$, 0.125µl

of 10 mmol/µl dNTP's, 0.25µl of 10µmolar Co1-2017F primer, 0.25µl of 10µmolar Co1-2017R primer, 0.125µl of Taq, and 19.5µl of nuclease-free H_2O. Reactions were amplified using the following parameters: 1 minute at 53˚C, 35 cycles of 10 seconds at 95˚C, 30 seconds at 55˚C, 1 minutes at 72˚C, with a final extension of five minutes at 72˚C.

PCR products were then run though gel electrophoresis to observe if DNA material was amplified (Fig. 3). Two different size gels were used based on the number of amplification samples being run. Small gels were run on a FisherBiotech Mini-Horizontal Electrophoresis System Unit (FB-SB-710) and the gel was created using methods listed in Appendix B. Medium gels were run on a FisherBiotech Midi-Horizontal Electrophoresis System Unit (FB-SB-1316) and the gel was created using methods in Appendix C. Fisher BioReagents Tris-Borate-EDTA (TBE), (10x Solution was diluted to a 0.5x concentration) and Fisher BioReagents Agarose (BP160-100) was used to make the gels. A Thermo Scientific EC 300 XL power supply was used for running a current through the gels. BioLine HyperLadder II (Cat. No. BIO-33039) and Qiagen GelPilot Loading Dye, 5x were used for band length analysis. A BioRad MyCycler (MyCycler version 1.065-000016) was used for all amplifications. Gels were observed in a Fotodyne incorporated FOTO/Analyst® Investigator FX using an Ethidium Bromide Filter and the FOTO/Analyst® PC Image version 10.41 software (Hartland Wisconsin).

Anastatus reduvii

Dried *A.reduvii* (Fig. 4) specimens were supplied by the USDA ARS Beneficial Insects Introduction Research Unit, Newark DE, from lab reared colonies maintained on *Halyomorpha halys*. Ten whole-body female specimens were extracted with a three-hour incubation at 56˚C for step 2 (Appendix A). PCR method 1 was used. Amplifications were confirmed by running a gel electrophoresis from each extracted sample and compared to the HyperLadder also used in the gel.

Another five extractions were conducted with a single middle leg from *A. reduvii* adult female specimens. They were incubated for three hours at 56˚ C (Appendix A). PCR method 1 was used. Amplifications were confirmed by running a gel electrophoresis from each extracted sample and compared to the HyperLadder also used in the gel.

Anastatus orientalis

Five right middle leg extractions were conducted with *A. orientalis* females supplied in 95% alcohol from a rearing colony of SLF at the USDA APHIS Buzzards Bay, MA, facility. Specimens were dried for 15 minutes on tissues before extractions were conducted. Extractions were incubated for three hours at 56˚C during step 2 of the extraction protocol (Appendix A). PCR method 1 was used, PCR cleanup was conducted (Appendix D) and samples were sent to DBI for sequencing.

A second attempt at extracting *A. orientalis* was conducted with slight modifications. Two female specimens were used by extracting a single middle leg, and two whole body extractions were used to test if a single leg was enough material for a successful extraction. Specimens were dried for 15 minutes on tissues before extractions were conducted. Extractions were incubated for 3 hours at 56˚C during

step 2 of the extraction protocol (Appendix A). PCR method 1 was used for both whole body and right leg extractions. PCR cleanup was conducted (Appendix D) and samples were sent to DBI for sequencing.

Five whole body extractions for female *A. orientalis* were conducted (Appendix A) and were incubated for 24 hours at 56˚C during step 2. From these extractions' PCR methods 1, 2, 3, and 4 were used.

Anastatus disparis (Ruschka)

Three adult male and three adult female *Anastatus disparis* (Fig. 5) were acquired from the USDA Beneficial Insects Introduction Research Unit in Newark. Specimens were collected by rearing from gypsy moth eggs in 1978 and were point mounted. Each pointed specimen was pinned into a piece of foam and placed in a small Tupperware container with damp paper towels. They were left in the container for 5 hours, making the specimens pliable enough to remove a single middle leg without destroying the entire specimen. Each middle leg was extracted using the Qiagen extraction protocol (Appendix A) and were incubated for 24 hours at 56˚C for step 2.

PCR methods 1, 2, 3, and 4 were used and each PCR product was run through a gel to look for the presence of DNA fragments. One lane of the gel included an extraction from *Anastatus reduvii* as a control to make sure the extractions and amplifications worked properly.

Anastatus mirabilis (Walsh and Riley)

Five adult female *Anastatus mirabilis* in 95% alcohol were acquired from the USDA Beneficial Insects Introduction Research Unit in Newark. Specimens were collected in May of 2010 by rearing from field-collected *H. halys* eggs. Specimens

were dried for 15 minutes on tissues before a single leg was removed for extraction. Extractions were incubated for 24 hours at 56˚C during step 2 of the extraction protocol (Appendix A). PCR methods 1, 2, 3, and 4 were used and each PCR product was run through a gel to confirm the presence of DNA fragments. In one well of the gel an extraction from *Anastatus reduvii* was used as a control to ensure the DNA was extracted and amplified properly.

Anastatus pearsalli (Ashmead)

Five adult female *Anastatus pearsalli* in 95% alcohol were acquired from the USDA Beneficial Insects Introduction Research Unit in Newark. Specimens were collected in May of 2008 by rearing from field-collected *H. halys*. Specimens were dried for 15 minutes on tissues before a single leg was removed for extraction. Extractions were incubated for 24 hours at 56˚C during step 2 of the extraction protocol (Appendix A). PCR methods 1, 2, 3, and 4 were used and each PCR product was run through a gel to look for the presence of sequences. In one well of the gel an extraction from *Anastatus reduvii* was used as a control to ensure the DNA was extracted and amplified properly.

Each sequence was then viewed in FinchTV version 1.4.0 (Geospiza Inc.) for sequence strength. Viable sequences were then trimmed on both the leading and trailing end to remove any poor base calls. Base calls that were coded as R, Y, and M were changed to the base with the highest call strength per location. Each pair (forward and reverse) of sequences was then aligned in MEGA version X (Kumar et al. 2018) using the ClustalW algorithm. Each separate alignment per species was then aligned in the same fashion. All alignments for each species were then analyzed for differences using the compute pairwise distances function of MEGA version X. Based

on the value of this test a suitable genetic sequence was chosen as an identification sequence for the species.

Table 2. *Anastatus* (Hymenoptera: Eupelmidae) species extracted with method

Species	Females	Males	Whole body or leg	Lyse time	PCR methods
Anastatus reduvii	10	0	Whole body	3 hours	1
Anastatus reduvii	5	0	Single leg	3 hours	1
Anastatus orientalis	5	0	Single leg	3 hours	1
Anastatus orientalis	2	0	Single leg	3 hours	1
Anastatus orientalis	2	0	Whole body	3 hours	1
Anastatus orientalis	5	0	Whole body	24 hours	1,2,3,4
Anastatus disparis	3	3	Single leg	24 hours	1,2,3,4
Anastatus mirabilis	5	0	Single leg	24 Hours	1,2,3,4
Anastatus pearsalli	5	0	Single leg	24 Hours	1,2,3,4

(PCR methods (1,2,3,4) listed in methods section of chapter 2)

2.3 Results

Anastatus reduvii was sequenced using the primers LCO1490 and HCO2098 and the PCR protocol from method number 1. Six of the ten whole body extractions had suitable sequence strengths. One was contaminated in the lab and three resulting in poor sequence quality. The six that were of adequate quality ranged in length (post

trimming) from 576-598bp. Three of the five single leg extractions had suitable sequence strengths with the remaining two having many missed base pair calls. The range (post trimming) for single leg extractions was 549-566bp in length.

All viable whole body (six) and single leg (three) extractions were then aligned using ClustalW algorithms in MEGA version X. After alignment, a pairwise distance test was run for all nine sequences (Table 3).

Table 3. Pairwise distance for *Anastatus reduvii* CO1 sequences

	WB1	*WB2*	*WB3*	*WB4*	*WB5*	*WB6*	*SL1*	*SL2*	*SL3*
WB1									
WB2	0.00167								
WB3	0.00251	0.00000							
WB4	0.00251	0.00252	0.00335						
WB5	0.00083	0.00083	0.00167	0.00083					
WB6	0.06212	0.06330	0.06425	0.06327	0.06330				
SL1	0.00176	0.00266	0.00354	0.00266	0.00266	0.06347			
SL2	0.00091	0.00182	0.00274	0.00182	0.00182	0.06328	0.00091		
SL3	0.00091	0.0018	0.00276	0.00184	0.00184	0.06380	0.00000	0.00000	

(WB=Whole Body; SL=Single Leg)

WB2 and WB3 sequences were found to have no differences between them and were longer sequences than the similar single leg extraction sequences, so this sequence was chosen as the representative CO1 sequence for *Anastatus reduvii* (Appendix E.). It is 598 base pairs in length.

Anastatus orientalis, A. disparis, A. mirabilis and *A. pearsalli* extractions all failed to produce fragments on the gels for unknown reasons. All PCR methods were

attempted for each species with a minimum of 5 runs for each species. The HyperLadder as well as *Anastatus reduvii* controls were visible on all the gels.

2.4 Discussion

While the CO1 gene of *Anastatus reduvii* was successfully sequenced, attempts to sequence all other *Anastatus* species were unsuccessful. This may have been due to specimen collection methods, age of specimens or variability within the CO1 gene within the genus. New custom primers targeting the CO1 gene should be a focus for future work on this genus as well as targeting other genes for analysis.

Other genes that have been used to separate families and subfamilies of the Chalcidoidea that might be beneficial for separating species within *Anastatus* include Ribosomal 18S (2105 bp) and 28S D2-D5 (1812 bp). Both have been successfully used, and might be a viable alternative to CO1 (Munro et al. 2011, Heraty et al. 2013). The molecular data from these genes have been used to support the monophyly of Diaprioidea, Mymarommatoidea and Chalcidoidea within the Proctotrupomorpha, which is the sister group of Chalcidoidea (Munro et al. 2011).

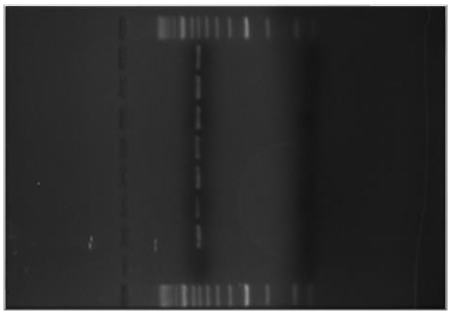

Figure 3. Gel with DNA banding from *Anastatus reduvii* extraction

Figure 4. Adult female *Anastatus reduvii*

Figure 5. Adult female *Anastatus disparis*

Chapter 3

TAXONOMY OF FOCAL HEMIPTERA AND REARING TECHNIQUES OF SEVERAL NATIVE HEMIPTERAN SPECIES TO BE USED IN NON-TARGET BIOLOGICAL CONTROL TESTS

3.1 Family Choices for Non-Targets species

For biological control to be utilized for the control of an invasive species several factors need to be considered. One factor is the potential impact of the control agent on native species (known as non-targets). Non-target species to be evaluated for SLF biocontrol were selected based on several criteria including geographical range, possibility of acquisition, similar biology and phylogenetic relationship to SLF. SLF is one of the largest North American planthoppers and completes most of its life history on aboveground portions of woody plants including trees and vines. SLF oviposits in groups of 30-50 eggs which are then covered with a waxy material. Egg masses are laid on vertical surfaces including trees, but also other structures such as telephone poles (Kim, Lee, et al. 2011). Because SLF is a Hemiptera, other species of this Order are the primary focus for non-target evaluations. The major focus has been placed on Fulgoromorpha species but species in Cicadomorpha and Heteroptera are included for diverse coverage.

The order Hemiptera within the class Insecta consists of approximately 100,000 named species (Bartlett et al. 2018) and is currently divided into four suborders. Of these, Coleorrhyncha (moss bugs) is confined to the southern hemisphere. The remaining three suborders consist of the Sternorrhyncha (aphids, psyllids, scale insects and allies), Heteroptera (true bugs) and Auchenorrhyncha (true hoppers). Auchenorrhyncha is further broken down into two infraorders, the

Cicadomorpha (leafhoppers, treehoppers, spittlebugs, cicadas and allies) and the Fulgoromorpha (planthoppers). The infraorder Fulgoromorpha contains a single extant superfamily (Fulgoroidea) and 21 extant families, of which 13 occur north of Mexico. The systematics of the superfamily has been investigated at the family level (Urban and Cryan 2007, Song and Liang 2013), but familial relationships remain insufficiently resolved.

3.2 Selection of Non-target Species for Collection and Evaluation

Fulgoroidea

Among North American planthopper families, Cixiidae and Delphacidae are basal on the tree, followed by the 'intermediate' families of Kinnaridae, Achilidae and Derbidae; then the 'higher Fulgoroidea' consisting of the remaining families Acanaloniidae, Caliscelidae, Flatidae, Issidae, Nogodinidae and Tropiduchidae. The Fulgoridae and Dictyopharidae are sister groups at the base of the 'higher Fulgoroidea (Urban and Cryan 2007)

The planthopper fauna north of Mexico consists of 12 families, 167 genera and 914 species (Bartlett et al. 2014), plus the subsequently reported adventive genus *Colpoptera* (Halbert 2010) (Nogodinidae, a 13th family); Including SLF, five additional adventive species have also been added to the North American planthopper fauna (*Petrusa epilepsis* (Kirkaldy) (Flatidae), *Tarophagus colocasiae* (Matsumura) (Delphacidae), *Opiconsiva tangira* (Matsumura) (Delphacidae), *Megamelus scutellaris* (Berg) (Delphacidae), and one other delphacid that was found but unreported (Tipping et al. 2014, Bartlett et al. 2015, Halbert and Bartlett 2015, Halbert 2016, Bahder et al. 2019). There are 74 genera and 259 species in the Northeastern

United States (Virginia, north to Maine), and 102 genera and 385 species in the Southeast. Pennsylvania has 51 genera and 115 reported species of planthoppers in 10 families (Barringer and Bartlett 2018). Planthopper species for host suitability studies were chosen among Eastern US taxa that are large-bodied, overwinter as eggs that might be accessible to parasitoids, and which might be readily collected in the field. We excluded from consideration the families Cixiidae, Achilidae, Derbidae (subterranean eggs and nymphs), Delphacidae, Caliscelidae (small size), Kinnaridae (small size, no local taxa), Tropiduchidae and Nogodinidae (no local taxa); leaving local taxa in the Acanaloniidae, Dictyopharidae, Flatidae, Fulgoridae and Issidae as containing the most likely candidates for host suitability studies. Focusing only on eastern species (since SLF is currently only found in the east) and with the removal of adventive species, 51 species remained for consideration. Most of these species are known or assumed to be univoltine and overwinter as eggs.

Other Hemiptera

During the last field season of research (summer 2019) several other species outside of the infraorder Fulgoromorpha were added to the USDA host range evaluation list. Within the infraorder Cicadomorpha of Auchenorrhyncha, which consists of 3,391 species in 412 genera in the Nearctic region (Bartlett et al. 2018), the family Membracidae was chosen for study. Membracidae is a very species-rich family within the United States (Deitz and Wallace 2012), with many species overwintering as eggs inserted into plant tissue. The species *Thelia bimaculata* (Fabricius) was chosen because it is locally common, large bodied, and is potentially impacted by SLF egg parasitoid.

Several species of Heteroptera (true bugs) were also chosen for rearing. There are over 3,830 species of Heteroptera reported north of Mexico, within seven infraorders, ~680 genera and 45 families (Henry & Froeschner, 1988)

The species chosen were selected based on their geographical range and life histories with a specific focus on how and when they lay their eggs, as well as egg size. It was also a consideration that native *Anastatus* are reported to parasitize the heteropterans selected for rearing (Mitchell and Mitchell 1983). Similar ecology and egg sizes to SLF could result in several Heteropteran species being targeted by biological control agents, including those within the families Reduviidae (assassin bugs) and Coreidae (leaf-footed bugs). There are roughly 150 species in 49 genera and 11 subfamilies of Reduviidae north of Mexico (Swanson 2018). Coreidae comprises 88 species in 33 genera north of Mexico (Henry 2009).

3.3 Life History and Ecology of Non-Target Taxa

Suborder Auchenorrhyncha, Infraorder Fulgoromorpha

Distribution and occurrence numbers for the suborder Auchenorrhyncha within the United States follows Bartlett et al. 2014.

Family Fulgoridae

Fulgoridae is represented North of Mexico by 10 genera and 18 species (including SLF). Most Fulgoridae are southwestern, with only a few species represented in the east (mostly southeast). Many species feed on trees and woody

shrubs, but a few taxa are grass feeders (Wilson and Wheeler 1992). There are five species represented in the east: *Amycle vernalis* (Manee), *Calyptoproctus marmoratus* (= *Alphina glauca* Auctt), *Cyrpoptus belfragei* (Stål), *C. reineckei* (Van Duzee) and *Poblicia fuliginosa* (Olivier).

Calyptoproctus marmoratus is a common species in the southern United States (north to Virginia). It is associated, through specimen data, with deciduous woods (Hoffman 2004), but the biology of the species has not been investigated and no host plant associations have been reported. *Poblicia fuliginosa* (Fig 6.) is widespread in the south (north into coastal Maryland) and feeds on winged sumac (*Rhus copallinum* L.) but may feed on other woody plants. The life history has not been investigated. Both species were focused on for this study due to locality, size and primary food source.

Amycle vernalis and the two species of *Cyrpoptus* apparently feed on grass, including the adventive African weeping lovegrass, *Eragrostis curvula* (Wilson and Wheeler 2005, 2010). *Crypoptus belfragei* is recorded as far north as northern Delaware and may overwinter as a late instar nymph. Wilson and Wheeler (2005) were unable to discern voltinism. *Crypoptus reineckei* and *Amycle vernalis* are southern species and are both reported as bivoltine by Wilson and Wheeler (2005). *Amycle vernalis* is rarely encountered (in South Carolina), and was observed to overwinter as late instar nymphs. *Crypoptus reineckei* is also reported to overwinter as late instar nymphs (in Florida and Georgia) by Wilson and Wheeler (2005). All three species were looked for, but not focused on due to differences in overwintering.

Family Dictyopharidae

Dictyopharidae are the sister group to the Fulgoridae and share similar biology. In the eastern United States, there are 21 species recorded (Bartlett et al., 2014). The most prominent species in the Mid-Atlantic region are *Scolops sulcipes* (Say), *S. perdix* (Uhler), *S. pungens* (Germar), *Rhynchomitra microrhina* (Walker), *R. lingula* (Van Duzee), and perhaps *Phylloscelis atra* (Germar) (the 'cranberry toad bug'). Most species are associated with dicots of a variety of families and are reported to be monophagous. *Scolops sulcipes* (Fig. 7) is one of the more numerous species in the Mid-Atlantic. *Rhynchomitra* species are reported on woody shrubs, and *Rhynchomitra microrhina* (Fig. 8) has also been reported on *Eragrostis curvula* (Schrad) (Wilson and Wheeler 2005). All three species were selected for this study due to locality, size, primary food source, life histories and possibility of acquisition.

Family Flatidae

Flatidae includes several abundant and polyphagous species on woody shrubs and semi-woody herbs. Three common flatids occurring in the Mid-Atlantic are *Flatormenis proxima* (Walker), *Metcalfa pruinosa* (Say) (the citrus flatid planthopper) and *Ormenoides venusta* (Melichar). All three species are polyphagous on woody plants and semi-woody plants, have a single generation a year, and overwinter as eggs (Bartlett et al. 2014).Wilson and McPherson (1981) attempted to rear all three species. *Flatormenis proxima* (Fig. 9) adults and nymphs were maintained and reared on green beans, *Phaseolus vulgaris* L., which also served as an oviposition site. *M. pruinosa* (Fig. 10) adults and nymphs were maintained and reared on green beans and on leaflets and twigs with attached leaves of black walnut (*Juglans nigra* L.) for adults.

Ormenoides venusta (Fig. 11) adults and nymphs were maintained and reared on green beans, paw paw leaves, *Asimina triloba* L., redbud leaves (*Cercis canadensis* L.) and black walnut leaflets and twigs with attached leaves for adults (Wilson and McPherson 1981a). These three species were chosen for this study due to locality, size, primary food source, life histories and ease of acquisition.

Family Acanaloniidae

Acanaloniidae includes two common species in the Mid-Atlantic (*Acanalonia conica* (Say), *A. bivittata* (Melichar)) and one uncommon species (*A. servillei* (Spinola)). *Acanalonia conica* (Fig. 12) and *A. bivittata* (Fig. 13) are polyphagous on a variety of woody and semi-woody plants, and are both univoltine (Wilson and McPherson 1981b). Both species overwinter as eggs, and deposit eggs directly into plant material. Rearing and egg acquisition has been attempted for both species with moderate success (Wilson and McPherson 1981b). Adults and nymphs of *A. bivittata* were collected and maintained as nymphs and adults on green beans and black walnut. Eggs were laid in the plants and visually detected by a row of waxy tufts on the wood where the females carve into the plant. The eggs are elongate, oval and white when laid. They turn an orange color six weeks before hatching. The chorion is translucent and has a polygonal sculptured pattern. The cephalic end has an elongate cylindrical process (Wilson and McPherson 1981b). *Acanalonia* nymphs and adults were reared and maintained on green bean and redbud, *Cercis canadensis. Acanalonia conica* eggs were found by locating a similar waxy tuft indicating their presence. Eggs are elongate and oval with a white coloration when laid, but turn orange six weeks before hatching. They have a translucent chorion with a polygonal sculpturing pattern and an elongate

process on the cephalic end (Wilson and McPherson 1981b). Both species were chosen for this research due to locality, size, life histories and primary food sources.

Family Issidae

Issidae include three species in the Mid-Atlantic, two within the genus *Thionia* and one recently placed within the genus *Aplos* (Gnezdilov 2018). *Aplos simplex* (Germar) is apparently polyphagous (Wheeler, Jr. and Wilson 1988). *Thionia elliptica* (Germar) is an oak feeding issid and *Thionia bullata* (Say) feeds on pine. All three species appear to be univoltine and overwinter as eggs (Wheeler, Jr. and Wilson 1988). *Aplos simplex* (Fig. 14) was selected for this research due to locality, size, life history and primary food sources.

Suborder Auchenorrhyncha, Infraorder Cicadomorpha

Family Membracidae

The family Membracidae is a very species-rich family within the United States, with most species on woody plants, especially oaks (Deitz and Wallace 2012). For the eastern United States, the highest reported number of species are reported from New York with 118, while the lowest number are in West Virginia with 14 species reported (Deitz and Wallace 2012). *Thelia bimaculata* (Fig. 15) is a large species that is commonly encountered locally and was a focal species for this study. The species is commonly found on *Robinia pseudoacacia* where populations persist into the fall. It has been documented (Funkhouser 1915) that this species deposits its eggs lower on the trunk of trees, carving slits into the plant material and depositing them in a palmate formation. The number of eggs can range from 3-6; it was reported that the tips of the

eggs are sometimes visible from the outside of the plant. Easy access to the eggs can lead to parasitism, and egg parasitism was recorded but none of the parasitoids were identified (Funkhouser 1915).

Suborder Heteroptera

Heteropteran species were chosen based on availability during collecting events. All species focused on for this research were acquired while doing general collecting for other Hemipterans.

Family Reduviidae

Arilus cristatus (Linnaeus) (wheel bug) is one of two species collected for research. It is univoltine, overwinters as eggs, is large and lays its eggs in clusters on trees in a similar manner as SLF. It is also a known of host for *Anastatus* (Hagerty and McPherson 2000). Hagerty and McPherson (2000) documented the life history of the species and rearing techniques. They reported that many of the egg clusters were parasitized with one of the parasitoids being *Anastatus reduvii*. They also collected females that laid eggs that were not exposed to a cold period. Those eggs hatched, signifying that a cold period is not required for egg development (Hagerty and McPherson 2000).

During the final field season three *Pselliopus barberi* (Davis) were collected while searching for *Arilus cristatus* and were kept alive in the lab for life history studies and to see if eggs would be acquired. Some research has been done on *Pselliopus barberi,* with life history reports of overwintering as adults (Swadener and Yonke 1975). This differs from all other species worked on during this research, but it

has also been reported that egg laying occurs between April and June. While this does not overlap with SLF egg laying, it could still present a target for early emerging parasitoids. Nymphs of *Pselliopus barberi* could also be targeted by nymphal parasitoids since both *Pselliopus barberi* and SLF nymphs would be occurring concurrently. Nymphal development from first instar to adult takes 59-91 days. Swadener and Yonke (1975) reported parasitism of an adult male which had an adult by *Xanthomelanodes arcuatus* (Say) (Diptera: Tachinidae) which emerged from its abdomen in September 1971.

Family Coreidae

Two species of Coreidae were collected for rearing studies. *Anasa tristis* (De Geer) (squash bug) is a highly researched species occurring throughout the United States, Mexico, Central America, and Canada. It is a pest of several cucurbit crops, and several studies focused on the life history of the species (Cornelius 2017). *Anasa tristis* (Fig. 16) develops from first instar to adult on pumpkin, *Cucurbita pepo* L. (Bonjour and Fargo 1989). It has also been shown that 2nd and 3rd instar *Anasa tristis* weigh more when feeding on *Cucurbita pepo* compared to *Cucumis sativus* L. (cucumber) (Cook and Neal 1999). Developmental rates at different temperatures have also been studied. No eclosion occurred at 15.6˚C, showing it to be below the developmental threshold for *Anasa tristis*. Eclosion also did not occur at temperatures greater than 33.3˚C, showing the upper lethal limit for development (Fargo and Bonjour 1988). Females prefer to oviposit on a cultivar they were reared on, but if they were not exposed to a specific cultivar no preference was shown (Cornelius 2017).

Leptoglossus fulvicornis (Westwood) was the second Coreidae species selected for this research. Its life history and rearing was reported by Wheeler and Miller (1990). It has been reported as far north as Ontario and south into Florida, and west to Texas. Its primary hosts are magnolia trees, including *M. virginiana* L., *M. grandiflora* L., *M. macrophylla* (Michx), *M. kobus* (DC.), *M. tripetala* L., *M. soulangiana* (Soul.-Bod.), *M.* x *loebneri* (Kache), *M. stellate* ((Siebold & Zuccarini) Maximowicz), and *M. heptapeta* ((Buc'hoz) Dandy). Adults overwinter in leaf litter surrounding magnolia trees after sub-freezing night temperatures occur. In Pennsylvania adults were recorded colonizing *Magnolia* during mid-June to early July when small fruits started to appear. Eggs were found as early as June 21st, but more frequently in July. Eggs were laid in linear chains on the foliage of the plant (Fig. 17), often along the midrib on the lower surface but sometimes along the edge of the leaf on either surface. Double chains were observed on rare occasions. Nymphal hatching occurred on July 5th but they did not initially feed. They aggregated around the egg mass and then moved to looser aggregations on leaves near the egg cluster. Third instars were observed as early as July 12th, and fourth and fifth instars were observed on July 25th. Adults were first observed on August 9th, with all other instars still present. Nymphs and adults fed on the fruits of the magnolia trees as well as the seeds that later protruded from the fruits of *M. tripetala*. *Leptoglossis fulvicornis* was reported to be difficult to rear in the laboratory. Only 10 adults were reared from eggs, with a mean total development period of 42.1 days. The newly hatched nymphs were placed in large plastic boxes with fresh water and pieces of magnolia fruit (Wheeler and Miller 1990).

Egg parasitism of *L. fulvicornis* has been reported by several species of Hymenoptera. *Gryon pennsylvanicum* (Ashmead) was collected from *L. fulvicornis* eggs from Georgia (Masner 1983) and a species of *Anastatus* identified as *Anastatus reduvii* was recorded parasitizing eggs of *L. fulvicornis* (Mitchell and Mitchell 1983).

3.4 Materials and Methods

Field collecting was conducted in several states in the Eastern United States for focus species (Table 4). Field collecting methods are given in Appendix F. *Poblicia fuliginosa* was field collected on *Rhus copallinum* in Bladen County, North Carolina from 2017 to 2019. *Acanalonia conica, Acanalonia bivittate, Flatormenis proxima, Metcalfa pruinosa, Ormenoides venusta, Rhynchomitra microrhina,* and *Scolops sulcipes,* and were field collected in Delaware from 2017 to 2019. *Aplos simplex* was field collected on *Robina pseudoacacia* in Delaware from 2017-2019. *Anasa tristis* was field collected in Delaware during 2019. *Leptoglossus fulvicornis* was field collected on *Magnolia virginiana* in Delaware in 2019. *Thelia bimaculata* was field collected in Delaware off *Robina pseudoacacia* in 2019. *Arilus cristatus* was not collected from the field but eight adults were donated for research in 2019 from field collecting. *Pselliopus barberi* was field collected in Delaware in 2019.

Table 4. Species selected for non-target host suitability studies

Family	Species (Common Name)	Plant association	Collection Site	Reared
Fulgoridae	*Poblicia fuliginosa*	*Rhus copallinum* (Winged Sumac)	Field collected (Bladen, NC)	Yes
Fulgoridae	*Calyptoproctus marmoratus*	Unknown (assumed as woody plants)	Not Found	No
Fulgoridae	*Crypoptus belfragei*		Not Found	No
Dictyopharidae	*Rhynchomitra microrhina*	Woody plants and *Eragrostis curvula* (Weeping lovegrass)	Field collected (Newark, DE)	Yes
Dictyopharidae	*Scolops sulcipes* (Partridge Bug)	Unknown (assumed dicot plants)	Field Collected (Hockessin, DE)	No
Flatidae	*Flatormenis proxima* (Northern Flatid Planthopper)	Polyphagous	Field collected (Newark, DE)	Yes
Flatidae	*Metcalfa pruinosa* (Citrus Flatid Planthopper)	Polyphagous	Field collected (Newark, DE)	No
Flatidae	*Ormenoides venusta*	Polyphagous	Field collected (Newark, DE)	No
Acanaloniidae	*Acanalonia conica*	Polyphagous	Field collected (Newark, DE)	Yes
Acanaloniidae	*Acanalonia bivittata*	Polyphagous	Field collected (Newark, DE)	Yes
Issidae	*Aplos simplex*	*Robinia pseudoacacia* (Black Locust) *Cephalanthus occidentalis* (buttonbush)	Field collected (Newark, DE)	No
Membracidae	*Thelia bimaculata* (Locust Treehopper)	*Robinia pseudoacacia* (Black Locust)	Field collected (Newark, DE)	Yes
Reduviidae	*Arilus cristatus* (Wheel Bug)	Predator	Donated	Yes
Reduviidae	*Pselliopus barberi*	Predator	Field collected (Newark, DE)	No
Coreidae	*Anasa tristis* (Squash Bug)	Polyphagous	Field collected (Newark, DE)	Yes
Coreidae	*Leptoglossus fulvicornis* (Magnolia leaf-footed bug)	*Magnolia spp.*	Field collected (Newark, DE)	No

Species rearing was conducted in an ambient (not temperature controlled) greenhouse or laboratory at the University of Delaware (Table 5). Rearing and egg collection methods are given in Appendix G. *Poblicia fuliginosa* was reared on *Rhus copallinum* (Fig. 18). *Flatormenis proxima, Metcalfa pruinosa, Ormenoides venusta, Aplos simplex,* and *Thelia bimaculata* were reared on *Robina pseudoacacia* (Fig. 19), and *Rhynchomitra microrhina* and *Scolops sulcipes* were reared on *Solidago* (goldenrod).The predatory species *Arilus cristatus* and *Pselliopus barberi* were reared in the laboratory and provided with various prey items. *Anasa tristis* was reared on *Cucurbita pepo* in both the laboratory and greenhouse. *Leptoglossus fulvicornis* was reared on *Magnolia virginiana* in the laboratory.

Table 5. Rearing techniques summary

Species	**Rearing Media**	**Housing**	**Location**
Poblicia fuliginosa	*Rhus copallinum* (Potted Plant)	12”x12”x24” mesh rearing cage (2017) 5’x2’x5’ mesh rearing cage (2018-2019)	Greenhouse (2018-2019)/Lab (2017)
Rhynchomitra microrhina	*Solidago* sp. (Planted)	12”x12”x24” mesh rearing cage (2017) 24”x24”x36” mesh rearing cage (2018-2019)	Greenhouse (2018-2019)/Lab (2017)
Scolops sulcipes	*Solidago* sp. (Planted)	12”x12”x24” mesh rearing cage (2017) 24”x24”x36” mesh rearing cage (2018-2019)	Greenhouse (2018-2019)/Lab (2017)
Flatormenis proxima	*Robina pseudoacacia* (Potted Plant)	24”x24”x36” mesh rearing cage	Greenhouse
Metcalfa pruinosa	*Robina pseudoacacia* (Potted Plant)	24”x24”x36” mesh rearing cage	Greenhouse
Ormenoides venusta	*Robina pseudoacacia* (Potted Plant)	24”x24”x36” mesh rearing cage	Greenhouse
Acanalonia conica	*Robina pseudoacacia*	24”x24”x36” mesh rearing cage	Greenhouse

Table 5. Continued

	(Potted Plant)		
Acanalonia bivittata	*Robina pseudoacacia* (Potted Plant)	24”x24”x36” mesh rearing cage	Greenhouse
Aplos simplex	*Robina pseudoacacia* (Potted Plant)	24”x24”x36” mesh rearing cage	Greenhouse
Thelia bimaculata	*Robina pseudoacacia* (Potted Plant)	24”x24”x36” mesh rearing cage	Greenhouse
Arilus cristatus	Various prey	24”x24”x36” mesh rearing cage	Lab (2019)/ Greenhouse (2019)
Pselliopus barberi	Various prey	Small rectangular plastic cages (‘kritter keeper’) and 1-quart container	Lab
Anasa tristis	*Cucurbita pepo* (Potted)	Small rectangular plastic cages (‘kritter keeper’) and 1-quart container	Lab
Leptoglossus fulvicornis	*Magnolia virginiana* (Cuttings)	12”x12”x24” metal rearing cage	Lab

3.5 Results

Of the targeted taxa, 14 species were field collected and returned to the laboratory for rearing (Table 6). The species were maintained under artificial conditions with carrying degrees of success, eventually securing eggs (the requisite life stage for *Anastatus orientalis* host suitability studies) for eight of the target taxa. Some of these eggs were sent to the USDA APHIS laboratory in Buzzard’s Bay, MA for host suitability testing, while others were retained for renewal of the colony; however, overwintering eggs during 2017-2018 and 2018-2019 winters was largely unsuccessful, apparently because of egg desiccation resulting from low humidity.

Table 6. Collection results for species targeted for host suitability studies

Family	Species	Collected	Years Found	Collection Locations	Eggs Acquired
Fulgoridae	*Calyptoproctus marmoratus*	No	n/a	n/a	n/a
Fulgoridae	*Crypoptus belfragei*	No	n/a	n/a	n/a
Fulgoridae	*Poblicia fuliginosa*	Yes	2017-2019	NC	Yes
Dictyopharidae	*Rhynchomitra microrhina*	Yes	2017-2019	DE	Yes
Dictyopharidae	*Scolops sulcipes*	Yes	2017-2019	DE	No
Flatidae	*Flatormenis proxima*	Yes	2017-2019	DE	Yes
Flatidae	*Metcalfa pruinosa*	Yes	2017-2019	DE	No
Flatidae	*Ormenoides venusta*	Yes	2017-2019	DE	No
Acanaloniidae	*Acanalonia conica*	Yes	2017-2019	DE	Yes
Acanaloniidae	*Acanalonia bivittata*	Yes	2017-2019	DE	Yes
Issidae	*Aplos simplex*	Yes	2017-2019	DE	No
Membracidae	*Thelia bimaculata*	Yes	2019	DE	Yes
Reduviidae	*Arilus cristatus*	Yes	2019	DE	Yes
Reduviidae	*Pselliopus barberi*	Yes	2019	DE	No
Coreidae	*Leptoglossus fulvicornis*	Yes	2019	DE	No
Coreidae	*Anasa tristis*	Yes	2019	DE	Yes

Family Fulgoridae

Poblicia fuliginosa was successfully collected during all field seasons (2017-2019) from Bladen County, North Carolina. The first collection trip (September 17th to September 21st) yielded 24 adults. The second collection trip (August 18th to August 23rd) yielded 28 nymphs and 20 adults, and the third collecting trip (August 24th to August 28th) yielded 11 nymphs [apparently 5th instar] and 30 adults. All locations were located either near a paved road or maintained sandy roads following power

lines. All collection sites consisted shrub/small tree edge habitat (plants mostly less than 10 feet tall) with sparse canopy cover. Direct sun caused the locations to be hot and sunlight was able to reach the soil in most areas. *Rhus copallinum* and *Pinus palustris* (Mill) (longleaf pine) were consistently the tallest trees within each collection location (Fig. 20). Smaller plants consisted of grasses and shrubs that often grew around the bases of the *Rhus copallinum* at some locations. The only standing water in any location occurred in roadside drainage ditches.

The exact number of nymphal instars for *Poblicia fuliginosa* is unknown (assumed to be 5^{th}) as well as stadia lengths. From collection dates during the research the only trip that did not yield nymphs was from September 17^{th} to September 21^{st}. The second and third collecting trips ranged from August 18^{th} to August 28^{th} and nymphs were collected. This shift in nymph appearances shows that *P. fuliginosa* must start reaching the adult stage by September, with the majority being adults by mid-September. Nymphs were often collected on smaller *R. copallinum* ranging from one foot to three feet in height. Many of the nymphs collected during this research were found within five inches from the ground on the trunk of the plant. More behavioral observations are given Appendix H.

During collection trips for *P. fuliginosa* it became apparent that their preferred host is *Rhus copallinum* but certain individual *Rhus copallinum* appeared more desirable than others. This “hot tree” or “favored tree” behavior has been observed in other fulgorids (Fig. 21). More information on “favored tree” behavior is given in Appendix H.

During the collection trips it also became apparent that trees that had been previously fed on by *P. fuliginosa* showed signs of the feeding. Both nymphs and

adults were frequently found within the first 12 inches of the base of the trunk. Around this area trees that had been previously fed on developed small nodules or bumps all the way around the trunk (Fig. 22). These nodules are assumed to be reactions made by the tree triggered by feeding. Older trees that were "favored" had multiple nodules up the trunk showing yearly growth and new feeding locations. Recent feeding events also were identifiable by sap flowing from the feeding location. Often sap would be seen coming from the feeding nodules of medium sized plants. While not all plants that had nodules or feeding wounds had *P. fuliginosa* on them, it was highly likely that individuals would be within the vicinity.

Collection locations were found on the third year of fieldwork (after the clearcutting of previous collecting locations) almost entirely by driving along roads and finding patches of *Rhus copallinum*. Once a patch was found the trees would be checked for feeding signs, and then further investigated if signs were present. When adults and nymphs were found, they were often discovered on trees where the main trunk was surrounded by plant material (often grasses or thorn bushes). Plant material would slowly be moved away from the trunk of the tree and adults would often be found within the bottom 5' of the trunk. The plant material is thought to provide shelter and reduce predation events. As many as five adults were collected from the same 10' of trunk covered in plant material.

Poblicia fuliginosa collected during the first field season (2017) were kept in a 12"x12"x24" mesh rearing cage in a laboratory without temperature control (Fig. 18). They were provided with *Rhus copallinum* cuttings roughly 18" tall. This was adequate to sustain the adults for several months, with the final adult dying on October 24th. *Poblicia fuliginosa* eggs had not previously been observed, but on October 12

eggs were deposited onto both the leaves and the branches of the *Rhus copallinum* cuttings provided. Eggs were laid in rows with 2-7 eggs per row. The collection of eggs was covered in a gray waxy material similar to SLF's oothecae (Fig. 23). Some egg masses lacked the protective covering which could be a result of the lack of resources by the female. Egg masses on the cuttings appeared to be cramped due to the usage of leaves as a surface for egg deposits. Eggs were left on the plant material and kept in the cage in the laboratory. The first egg hatched on February 15th, 2018, taking 126 days to develop with no cold period given. A second egg hatched the following day (February 16th, 2018). Both nymphs (Fig. 24) were placed in a 1-quart container with mesh attached to the lid. The nymphs were provided *Robinia pseudoacacia* cut from live plants kept in the greenhouse. A *Rhus copallinum* cutting from a plant located outside was also provided but the plant was senescing when collected. No feeding events were observed, and nymphs survived until March 8th, 2018 (20 days). One egg mass was also in a petri dish with filter paper as a base and sealed with parafilm wax. Two eggs from the egg mass started to hatch, but the nymphs were unable to exit the eggs completely and died. This was presumed to have happened due to a lack of humidity. All other eggs masses were placed in a refrigerator unit to halt further development and emergence until they could be sent to USDA APHIS cooperators for further studies.

During the second and third field season (2018/2019) collected nymphs and adults were placed in a larger rearing cage with potted *Rhus copallinum* in a greenhouse. The expectation was that life expectancy would go up and more eggs would be deposited on larger potted plants in a larger cage opposed to a small cage on cuttings. A total of 76 egg masses were laid during 2018. A large majority of them

were placed on the wooden boards used to construct the rearing cage, while a smaller amount was placed on the trees. One egg mass was laid on the top of the cage on the mesh material. This shows that *P. fuliginosa* can lay eggs on non-living surfaces, just as SLF has been observed laying eggs on a multitude of non-living surfaces. Several egg masses were removed from the cage and sent to the USDA APHIS facility for non-target host specificity testing with biological control agents. The remainder of the eggs were left in the rearing cage and kept in the greenhouse over the winter months. Since no cold period was required for the previous year's nymphs to emerge, it was expected that keeping the eggs warmer in the greenhouse might shorten the egg development time. No hatching events were observed, and no nymphs were seen until June 14th, 2019 when one late instar nymph (Fig. 25) was seen in the cage. It was located due to the honeydew it had secreted onto the leaves. The nymph eclosed on June 25th, 2019, which is significantly earlier in the year compared to field observations in North Carolina. One July 1st, 2019 eggs were removed from both the wooden beams and the trees to see why there were not more hatching events. All the eggs were completely desiccated and appeared collapsed. This is thought to have occurred due to an insufficient humidity in the greenhouse.

The 2019 colony of *P. fuliginosa* produced 57 egg masses by November 16th, 2019. During the following week ants chewed through the mesh at the base of the cage to access the potted plants. They killed all remaining adults (estimated 15) and removed all living eggs from their oothecas. While this was an isolated incident in a greenhouse, it shows that the eggs are likely not protected from predators such as ants in the wild.

Family Dictyopharidae

Rhynchomitra microrhina was found on the University of Delaware campus and the Ashland Nature Center in northern Delaware. At the University of Delaware, the main collection site was the wetlands study area located near Townsend Hall. Roughly 25 adults were collected from the wetland (Fig. 26) during the 2018 field season and 34 adults were collected during the 2019 field season. For the 2018 field season the first adult was collected on July 26th, 2018. No adults were found after October and no nymphs were found during collecting. For the 2019 field season adults were first collected on August 23rd and the last adults were collected on September 14th. No nymphs were found during the 2019 field season.

All adults were collected using a sweeping technique through asteracous plants. Plants targeted during collections included an unknown asteracous plant (possibly in genera *Aster* or *Symphyotrichum*) and *Solidago.* Most adults collected during both field seasons were collected from patches of these plants. Because of the number of adults collected from goldenrod it was used for rearing in the greenhouse. Feeding was also recorded on goldenrod, confirming it as a possible food source in the field for the species. Eggs for this species were previously undocumented, therefore the 2018 field collected adults were kept in an ambient-temperature laboratory for observation. No copulation was ever observed, and the last adult died on September 24th, 2018. However, on September 25th, 2018 nine eggs were discovered in the rearing cage. Seven of the eggs were deposited singly, each being laid at the base of a leaf on the node of the goldenrod near the main stem (Fig. 27). These seven eggs were not imbedded within the plant but were placed into the crease caused by the petiole branching from the node. Two eggs were deposited in a similar way at the top of the

plant were new growth was occurring. Because of this location, as the plant grew the eggs became embedded within a leaf. This is not believed to be the intended outcome for the eggs, but an unintended scenario due to the egg laying location.

Eggs are oval measuring 0.42-0.47 mm in length (Fig. 28). The chorion is slightly rugose with a light-yellow coloration. On one end of the oval there is a filamentous cap that is roughly 0.21 mm in length. Five eggs were sent to USDA APHIS for non-target testing and four eggs were kept for rearing Eggs were removed from plants and placed in a small petri dish filled halfway with soil. Eggs were placed directly onto the soil because it was hypothesized that eggs fall from the plants in the wild since they were not attached to the plant, and goldenrod is a perennial species which dies above ground during the winter season in Delaware. Several (3-5) drops of distilled water were applied to the soil every three days to maintain humidity and the petri dish was kept covered. The petri dish was placed within a small plastic container with a dish of water to maintain humidity within the larger container. The petri dish was not sealed closed with parafilm.

On April 23rd, 2019 three out of four eggs turned a light pink color and became noticeably smoother than the previous yellow stage (Fig. 29). The filamentous cap also began to retract from the tip forming a cage-like shape. On April 29th, 2019, the filamentous cape opened at the tip and each individual fiber spread outward in a star-shape (Fig. 30). The egg that did not undergo changes remained yellow and the filamentous cap remained straight. The first egg hatch occurred on April 30th, 2019 from one of the pink eggs (Fig. 31). The next two eggs that turned pink hatched on May 3rd, 2019 and May 7th, 2019, respectively.

All three nymphs were moved to a 1-quart container with a hole cut in the lid and mesh glued over it. The bottom 1.5 inches was filled with soil and a small goldenrod was planted. The container was watered every three days with a spray bottle so that the soil was not flooded. This was done to prevent any unseen nymphs from drowning. The first second instar *R. microrhina* nymph was observed on May 28th, 2019. One first instar nymph was found dead on June 3rd, 2019. The second surviving nymph was observed as a second instar on July 2nd, 2019. Both nymphs died before reaching third instar but survived until August 25th, 2019. This extended second instar death was possibly due to a low-quality food sources since the plant was small.

During the 2019 field season a larger rearing cage was placed in a greenhouse to provide a higher temperature for collected adults. A bin with soil was used instead of pots to allow for falling eggs to land in soil. All adults survived until October 11th, 2019. A total of 21 eggs were collected from the two plants, all laid in a similar way to the previous description except for one instance of two eggs laid next to each other. Most of the eggs were found semi-desiccated which is possibly a result of the greenhouse conditions. Eggs were moved from the plant material into the same petri dish setup used the year before.

Scolops sulcipes was collected from Ashland Nature Center using a sweep net for collection. Small patches of asteracous plants with little-to-no canopy cover throughout the center were surveyed (Fig. 32). Two patches were found to contain *S. sulcipes* during the 2018 field season, and one location was found to contain them during the 2019 field season. Ten adults were collected in 2018 and three adults in 2019. No nymphs were found during collections. There were no bodies of water near

the sweep netted fields, suggesting wetland habitat is not favored, unlike for *Rhynchomitra microrhina*.

During the 2018 field season all collected adult *S. sulcipes* were kept in a rearing cage in an ambient laboratory for close observation. Goldenrod was provided as a food source. Adults were observed with mouthparts inserted into both the main stem and the leaves. This observation confirms that goldenrod is a viable food source for *S. sulcipes*. Two adults were observed with their abdomens touching in a possible copulation on July 23rd, 2018. The last adult died on September 10th, 2018. All plants were visually inspected and deconstructed and the first 2" of soil was removed from the pots and inspected under a microscope. No eggs were found.

During the 2019 field season only three adult *S. sulcipes* were collected, all from Ashland Nature Center. They were all placed in a large rearing cage with a bin filled with soil and goldenrod, similar to the pots from the previous year. All plants were removed and tapped over white sorting trays to collect any loose eggs. Plants were then observed under a microscope for attached eggs and then cut open. The soil was also removed and observed for eggs. No eggs were found at any location.

Family Flatidae

Flatormenis proxima was collected from the University of Delaware, Ashland Nature Center and C&D Canal in northern Delaware. Sweeping plant material was the primary method of collection. During the 2018 field season a first instar nymph was found on June 6th. It was placed in a small container and molted to second instar the next day (June 7th, 2018). The first adult *Flatormenis proxima* collected in 2018 was on July 9th. Late instar nymphs were collected through July 26th, 2018, and

subsequently only adults were found. Over 100 individuals were collected during the field season. All instars were successfully reared to adult on *Robinia pseudoacacia* and adults were maintained in the greenhouse until October 10th, 2018. One tree was removed from the greenhouse and inspected for eggs. Several egg laying locations were found on the woody parts of the plant: however, none were found on that year's new growth. Eggs were laid singly in a longitudinal line with a minimum of four eggs and a maximum of 32 eggs per line (Fig. 33). Their description matches Wilson and McPherson (1981). All eggs in the tree that was cut apart were sent to USDA APHIS for non-target testing. The tree that was not dismantled was moved to a cooler greenhouse for overwintering. This tree contained 15 eggs, on which one hatching event was recorded on June 12th, 2019. Two other nymphs were found in the cage on June 14th, 2019. On June 25th, all nymphs were found dead.

The 2019 field season gave similar collection results as the 2018 field season. First instar nymphs were first seen on June 4th, 2019 and second instar nymphs were first seen on June 12th. Fourth instar nymphs were seen on June 25th and adults were first collected on July 1st, 2019. No nymphs were found after August 8th, 2019. Egg acquisition was increased for the final field season, with a single larger tree containing 107 egg in total. All 107 were sent to the USDA APHIS facility in Buzzards Bay.

Metcalfa pruinosa was collected from the University of Delaware, Ashland Nature Center and the C&D Canal. Sweeping *Rhus copallinum* 4' tall or less was the primary method of collection. During the 2018 field season the first instar nymphs were found starting on June 18th, 2018. Nymphs were found through August 10th, 2018. The first adult was found on July 9th, 2018. During the 2019 field season several first instar nymphs were found on July 7th, 2019. Second instar nymphs were found on

June 12th, and late instar nymphs (possible fourth or fifth) were found on June 25th, 2019. The first adult was collected on July 10th, 2019 at the C&D Canal. Over 100 individuals were collected during each field season.

All collected individuals were placed in rearing cages in the greenhouse during both years. Bark was hung in each cage and placed on each tree trunk to provide more space for egg laying. Nymphs and adults were maintained on *Rhus copallinum* with the last adult found dead on October 2nd, 2018. No eggs were collected during the 2018 or 2019 field season.

Ormenoides venusta was collected at the University of Delaware and the C&D Canal. Sweeping *Rhus copallinum* 4' tall or less was the primary method of collection. No nymphs were found during collecting. Seven adults were found during the 2018 field collecting season. The first collection date was July 11th, 2018 and the last adult was found on August 28th, 2018. For the 2019 field season the first adult was collected on July 26th, 2019 and the last adult collected was on August 25th, 2019.

All adults were maintained on *Rhus copallinum* within the greenhouse. The last *O. venusta* adult for the 2018 season died on September 10th, 2018 and the last adult from the 2019 field season died on September 24th, 2019. All plants from the 2018 field season were visually checked for eggs. No eggs were found and both plants were taken apart and cut open. No eggs were found within the plant.

Family Acanaloniidae

Acanalonia conica was collected from the University of Delaware, Ashland Nature Center, and the C&D Canal. Sweeping *Rhus copallinum* 4' tall or less was the primary method of collection. During the 2018 field season the first instar nymph was

found on June 12th, 2018. Second instar nymphs were seen on June 19th, 2018. Nymphs were present through August. The first adult was collected on July 9th, 2018. Adults were found until October 8th, 2018.

All nymphs and adults were maintained on *Rhus copallinum* in the greenhouse. Over 100 individuals were collected during the 2018 and 2019 field season. Adults collected during the 2018 field season survived until October 29th. Eggs were found on several branches of the trees. *Acanalonia conica* laid eggs in both the woody branches of the plant as well as the new growth for the season. Eggs were laid singly in longitudinal lines (Fig. 34) as described by Wilson and McPherson (1981). The number of eggs laid in each longitudinal strip ranged from eight to 27. Harder woody portions of the tree contained shorter egg strips than on the comparatively softer new growth. One plant contained roughly 40 eggs inserted into the plant material and was moved into the overwintering greenhouse. Several egg strips were on the new growth of the plant, some of which fell off during the winter. Weeds were allowed to grow in the pots to simulate weedy fields were nymphs were often found. Two first instar nymphs were found in the rearing cage on May 6th, 2019. No individuals were found again in the cage until July 15th, 2019 when three adults and one fifth instar nymph were found on the *Rhus copallinum* still in the cage. It is presumed that the nymphs spent a large majority of the time in the weedy pots where they were difficult to locate, and only moved to the *Rhus copallinum* after reaching a later stage in development. All reared *A. conica* were found dead on August 14th, 2019 and no eggs were found.

During the 2019 field season the first instar nymphs were found on June 4th, 2019. Second instar nymphs were first found on June 12th, 2019. Later instar nymphs (fourth or fifth) were found on June 25th. The first adult was collected on July 10th,

2019 from the C&D Canal. One tree was dismantled and contained more than 150 eggs. All eggs were laid in similar manner as in previous field seasons, and all eggs from that tree were sent to USDA APHIS. Another tree containing a similar amount of eggs was kept in the greenhouse for rearing purposes.

Acanalonia bivittata was found at the University of Delaware, Ashland Nature Center, and the C&D Canal. Sweeping asteracous fields was the main collecting method. *Acanalonia bivittata* preferred weedy fields with little to no canopy cover compared to *A. conica,* which was collected primarily from trees and plant material in field edge environments. During the 2018 field season the first instar nymphs were found on June 12th. One second instar nymph was found on June 27th, 2018 and four late instar (fourth or fifth) nymphs were collected on July 9th, 2018. The first adult was collected on July 19th, 2018 from Ashland Nature Center. A total of 27 individuals were collected during the 2018 field season.

All nymphs and adults were maintained on *Rhus copallinum* in the greenhouse. The last adult died on September 27th, 2018 and eggs were found on the plants. Eggs were only laid on the new growth for that season (Fig. 35), and no eggs were placed in the woody material of the plant. Eggs were laid singly in longitudinal strips ranging from 11 to 21 eggs. One plant was cut into pieces and all eggs found, as well as all new growth that had fallen off the plant, were sent to USDA APHIS. The remaining plant containing roughly 23 eggs was moved to the overwintering greenhouse. Weeds were allowed to grow in the pots to provide more habitat for the nymphs if they hatched. Two nymphs were recorded emerging on May 3rd, 2019. Both nymphs molted to second instars on May 15th, 2019. No other eggs hatched and both nymphs were found dead on May 28th.

During the 2019 field season one first instar nymph was found on June 12th. Two second and third instar nymphs were collected on June 24th. Four late instar nymphs (fourth or fifth) were collected on July 7th. Ten adults were found at the C&D Canal on August 2nd and seven adults were collected from the University of Delaware on August 8th.

The only tree that contained *A. bivittata* was dismantled and branches containing eggs were sent to USDA APHIS. Roughly 60 eggs were collected from the tree, with most of them laid in the new growth as in previous field seasons. Several eggs were also laid in the thorns of the tree, which had not been observed previously. A total of 11 thorns contained eggs, in strips of 3-6. Five thorns contained two strips of eggs on opposite sides of the thorn (Fig. 36).

Family Issidae

Aplos simplex was collected from the University of Delaware. Sweeping *Rhus copallinum* was the primary collection method, but two individuals were collected on *Cephalanthus occidentalis* L. (common buttonbush). During the 2018 field season only two nymphs were collected. One was collected on July 9th on *Cephalanthus occidentalis* and was presumed to be fourth or fifth instar. The second nymph collected was found on July 16th, 2018 and was collected with an adult. The first adult collected was on July 9th, 2018 and must have molted recently because it was still teneral. Total of 26 *A. simplex* were collected during the 2018 field season, with the last one collected on August 16th, 2018.

Of the 26 collected *A. simplex,* 10 died during transportation back to the greenhouse for unknown reasons, as several other species being transported at the

same time did not die. It is possible that the stress of being collected caused this high die-off percentage. Of the remaining collected individuals during the 2018 field season, none survived for more than two weeks after being placed in their rearing cage with *Rhus copallinum*.

One plant used in the rearing cages was removed after the last adult had died. No eggs were found on the tree or in the soil (methods in Appendix G). The second tree was moved to a cooler greenhouse for overwintering. No emergence was observed from this tree and after the winter season the tree was also taken into the lab and inspected for eggs. No eggs were found on this tree either.

During the 2019 field season *Aplos simplex* was collected from the University of Delaware and the C&D Canal. A total of 26 individuals were collected. Seven third and fourth instar nymphs were collected from a single tree on June 27th, 2019. The first adult was collected on July 10th. The last adult was collected on August 8th. As a result of the high percentage of die-offs during the 2018 field season, collection trips for *A. simplex* during the 2019 field season were focused just on this species. Collection time was limited to two hours in the field before collected individuals were placed in the rearing cage, and no other species were collected during this time. This limited the time that individuals were in a collection jar and removed the sorting of species before they would be placed in cages. These methods had more favorable results with all seven nymphs eclosing as adults and no quick die-off events from the adults collected in the field. Nymphs were assumed to have eclosed as adults due to the lack of any dead nymphs found in the rearing cage after all adults had died. The last adult survived until September 16th, 2019.

No eggs were found from *Aplos simplex*, either on the plants or in the soil after inspection.

Family Reduviidae

Arilus cristatus mating was observed between the first two donated individuals on September 30th, 2019. Each was fed twice (Fig. 37) before being placed in the same container. Mating occurred roughly 30 minutes after introduction with the female attached to the fine mesh placed underneath the lid of the enclosure. Eggs from this mated pair were laid 20 days later (October 20th, 2019). The mass contained 38 eggs in a cluster (Fig. 38) and two eggs laid away from the cluster. All eggs were attached to the fine mesh under the lid. The second male received through donation died two days later before it was introduced to a female.

All other females received were placed in separate rearing cages as described in the methods section for *A. cristatus*. Of the five females kept in this manner, four deposited eggs, three clustered on the mesh under the lid and one cluster on the paper towel used as refugia. The cluster on the paper towel contained 39 eggs. The three other egg clusters contained four, 28, and 183 eggs. Females died shortly after oviposition. Observed behaviors during rearing are described in Appendix H.

Pselliopus barberi eggs were not obtained during rearing, but this was expected due to the overwintering of adults and egg laying eggs during early spring. The two nymphs collected each molted once after collection. One eclosed as an adult 20 days after collection, and the other molted into a later instar 34 days after collection. The nymph that eclosed died 17 days after eclosion. The nymph that molted into a later instar is still alive and has not eclosed at this time. This could be

due to an inadequate amount of food provided, or possibly due to rearing conditions. The adult that was collected during the field season has survived and is still eating in rearing conditions. One-quart soup containers appear to be adequate enclosures for keeping individuals alive. Observed behaviors during rearing are described in Appendix H.

Family Coreidae

Leptoglossus fulvicornis nymphs and adults were kept in the same enclosure with cuttings of *Magnolia virginiana* that contained fruit. Both adults and nymphs were observed feeding on the fruit but were never observed feeding on the leaves or twigs. All three nymphs collected eclosed as adults, with the last eclosion on August 28th, 2019. The last adult died on October 11th, 2019 and no eggs were ever acquired from the colony.

The first *Anasa tristis* adults (collected in the halls of Townsend Hall on June 24th, 2019) were observed mating on a pot in their enclosure on July 3rd. A cluster of 23 eggs was discovered on the underside of a *Cucurbita pepo* leaf on July 12th and they all hatched on July 25th. All nymphs remained around the cluster of eggs for three days before moving about on the plant. Shortly after hatching the plant and all nymphs as well as the two adults were moved into a mesh rearing cage in the greenhouse. Several nymphs and adults collected from the field were added to the colony as they were collected. One egg mass was found on August 8th, 2019 and contained 42 eggs (Fig. 39). It was laid on a leaf which subsequently fell off into bottom of the cage, where it was found desiccated a few days later. Another cluster of seven eggs was found attached to the cage mesh on August 11th and was also found desiccated a few

days later. The mesh cage with the colony was moved back into the lab where the first cluster of eggs was successfully hatched. No further egg cluster have been found.

3.6 Discussion

Of the 14 species that were studied, eggs were acquired from eight, and nymphs were hatched from six. Since all species are apparently univoltine in nature (in the northeast United States) only a small number of attempts at successful rearing are possible each year. Self-sustaining rearing colonies were not achieved for any species, and this needs to be a focus of future research. An adequate overwintering environment needs to be investigated for each species since egg desiccation appeared to be a significant issue for several species eggs when kept in greenhouse conditions. Larger self-sustaining colonies for each species would allow a consistent amount of eggs to be supplied to USDA APHIS facilities to assist in biological control research for SLF.

Figure 6. Adult *Poblicia fuliginosa*

Figure 7. Adult *Scolops sulcipes*

Figure 8. Adult *Rhynchomitra microrhina* on plant material in lab

Figure 9. Adult *Flatormenis proxima*

Figure 10. Two adult *Metcalfa pruinosa* photographed in the field

Figure 11. Seven adult *Ormenoides venusta* photographed by Charles Bartlett

Figure 12. Adult *Acanalonia conica*

Figure 13. Four adult *Acanalonia bivittata* on *Robinia pseudoacacia* in rearing cage

Figure 14. Adult *Aplos simplex*

Figure 15. Several *Thelia bimaculata* on *Robinia pseudoacacia* in the field in Delaware

Figure 16. Two adult *Anasa tristis* mating

Figure 17. Strip of Leptoglossus fulvicornis eggs on Magnolia virginiana

Figure 18. Rearing cage used for *Poblicia fuliginosa*

Figure 19. General rearing cage setup for Acanaloniidae, Issidae, Flatidae and Membracidae

Figure 20. Field collecting site for *Poblicia fuliginosa* in Bladen County, North Carolina

Figure 21. Single “favored tree” (*Rhus copallinum)* of *Poblicia fuliginosa* in Bladen County, North Carolina

Figure 22. Late instar *Poblicia fuliginosa* on *Rhus copallinum* with seeping feeding wound

Figure 23. *Poblicia fuliginosa* egg mass laid on a plastic pot

Figure 24. Lab hatched first instar *Poblicia fuliginosa*

Figure 25. Late instar *Poblicia fuliginosa* nymph hatched in the greenhouse

Figure 26. Collecting habitat for *Rhynchomitra microrhina* in Newark, Delaware

Figure 27. *Rhynchomitra microrhina* egg placed at base of petiole on *Solidago*

Figure 28. *Rhynchomitra microrhina* egg

Figure 29. *Rhynchomitra microrhina* egg after 210 days

Figure 30. *Rhynchomitra microrhina* egg after 211 days

Figure 31. First instar *Rhynchomitra microrhina* nymph hatched in lab

Figure 32. Collection site for *Scolops sulcipes* in Newark, Delaware

Figure 33. Strip of *Flatormenis proxima* eggs lain in *Robinia pseudoacacia*

Figure 34. Strip of *Acanalonia conica* eggs lain in *Robinia pseudoacacia*

Figure 35. Strip of *Acanalonia bivittata* eggs lain in *Robinia pseudoacacia*

Figure 36. Strip of *Acanalonia bivittata* eggs lain in a thorn on *Robinia pseudoacacia*

Figure 37. *Arilus cristatus* female eating an *Anasa tristis* nymph

Figure 38. Cluster of *Arilus cristatus* eggs laid on mesh lid of enclosure

Figure 39. Cluster of *Anasa tristis* eggs laid on *Cucurbita pepo*

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Appendix A

QIAGEN DNEASY BLOOD AND TISSUE KIT

1. For each specimen to be extracted, label three 1.5 ml microcentrifuge tubes, 1 DNEasy mini spin column, and one 0.5 ml microcentrifuge tube
2. Either remove specimens' right leg and clean off any excess alcohol with air drying and blotting or clean entire specimen with air drying and blotting
3. Place tissue in a 1.5 ml tube and break apart with a micro pestle
4. Add 180 µl Buffer ATL to each sample
    a. Vortex each sample for 15 seconds
5. Add 20 µl Proteinase K to each sample
    a. Vortex each sample for 15 seconds
6. Incubate samples at 56°C for a period of time (mentioned in methods)
    a. Vortex every hour
7. Remove cuticle if required
8. Vortex for 15 seconds
9. Add 200 µl Buffer AL
    a. Vortex for 15 seconds
10. Add 200 µl ethanol (96-100%)
    a. Vortex for 15 seconds

11. Pipet mixture (including precipitate) into the DNEasy mini-spin column
12. Centrifuge at ≥ 8,000 rpm for 1 minutes
    a. Discard flow-through and collection tube
13. Place mini-spin column in new collection tube
14. Add 500 µl Buffer AW1
15. Centrifuge at ≥ 8,000 rpm for 1 minute
    a. Discard flow-through and collection tube
16. Place mini-spin column in new collection tube
17. Add 500 µl Buffer AW2
18. Centrifuge at 14,000 rpm for 3 minutes
    a. Discard flow-through and collection tube
19. Place mini-spin into clean labeled 1.5 ml microcentrifuge tube
20. Add 200 µl Buffer AE directly to membrane
    a. Incubate at room temperature for 1 minute
21. Centrifuge at ≥ 8,000 rpm for 1 minute
22. Save 1.5 ml tube and place mini-spin in new labeled 1.5 ml tube
23. Centrifuge at ≥ 8,000 rpm for 1 minute
24. Combine elution's and store at -80°C for thermocycling and purification

Appendix B

SMALL GEL ELECTROPHORESIS METHODS

1. Pouring Gel
    a. To a 100 ml flask, add 50 ml 0.5xTBE + 0.75 Agarose
    b. Microwave on high for 30 seconds
        i. Swirl flask
    c. Microwave on high for 15 seconds
        i. Swirl flask
    d. Microwave on high for 10 seconds
    e. Let cool on workbench for 10 minutes
    f. Set up gel tray for pouring
        i. Wet rubber gaskets with distilled water
        ii. Slide tray into gel rig with opened sides against rig walls
    g. After agarose has cooled add 7.5 µl Ethidium Bromide
        i. Swirl flask
    h. Pour gel into tray and insert comb on one side in grooves
    i. Let sit for 20 minutes
    j. Remove gel tray and turn 90˚ and place back in rig
        i. Remove comb
    k. Wells should be closest to negative (black) terminal of rig
    l. Add enough 0.5xTBE to rig to cover gel
2. Loading Gel

a. Acquire 6x loading dye and HyperLadder
b. Add 1.0 µl 6x loading dye per 5.0 µl PCR product
c. Mix by finer vertexing then centrifuge
d. Load 5 µl HyperLadder into first and last lanes of gel
e. Load PCR product and loading dye mixtures into lanes
f. Place lid on rig with red and black nodes matching
g. Plug in electrodes to power supply

3. Running Gel
    a. Turn on power supply
    b. Run at 100 Volts for 60 minutes
    c. When completed turn off power supply
    d. Remove gel and tray
    e. Tilt tray to drain off excess TBE
    f. Remove gel from tray and place in Fotodyne FOTO/Analyst® Investigator FX
    g. Open FOTO/Analyst® PC Image Software and click acquire live image
    h. Turn on FOTO/Analyst® and push Trans button
    i. Adjust contrast until HyperLadder is visible

Appendix C

MEDIUM GEL ELECTROPHORESIS METHODS

4. Pouring Gel
    a. To a 250 ml flask, add 150 ml 0.5xTBE + 2.25 Agarose
    b. Microwave on high for 40 seconds
        i. Swirl flask
    c. Microwave on high for 25 seconds
        i. Swirl flask
    d. Microwave on high for 20 seconds
    e. Let cool on workbench for 15 minutes
    f. Set up gel tray for pouring
        i. Wet rubber gaskets with distilled water
        ii. Slide tray into gel rig with opened sides against rig walls
    g. After agarose has cooled add 20 µl Ethidium Bromide
        i. Swirl flask
    h. Pour gel into tray and insert comb on one side in grooves
    i. Let sit for 30 minutes
    j. Remove gel tray and turn 90˚ and place back in rig
        i. Remove comb
    k. Wells should be closest to negative (black) terminal of rig
    l. Add enough 0.5xTBE to rig to cover gel
5. Loading Gel

a. Acquire 6x loading dye and HyperLadder
b. Add 2.0 µl 6x loading dye per 10.0 µl PCR product
c. Mix by finer vertexing then centrifuge
d. Load 10 µl HyperLadder into first and last lanes of gel
e. Load PCR product and loading dye mixtures into lanes
f. Place lid on rig with red and black nodes matching
g. Plug in electrodes to power supply

6. Running Gel
    a. Turn on power supply
    b. Run at 100 Volts for 90 minutes
    c. When completed turn off power supply
    d. Remove gel and tray
    e. Tilt tray to drain off excess TBE
    f. Remove gel from tray and place in Fotodyne FOTO/Analyst® Investigator FX
    g. Open FOTO/Analyst® PC Image Software and click acquire live image
    h. Turn on FOTO/Analyst® and push Trans button
    i. Adjust contrast until HyperLadder is visible

Appendix D

QIAGEN PCR PURIFICATION METHODS

1. Add 50 µl Buffer PB to 10 µl PCR reaction mix (result of thermocycle)
2. Place mini-spin into a collection tube
3. Centrifuge for 60 seconds at 13,000 rpm
4. Discard flow through and place mini-spin in same collection tube
5. Add 750 µl Buffer PE
6. Centrifuge for 60 seconds at 13,000 rpm
7. Discard flow through and place mini-spin in same collection tube
8. Centrifuge for 1 minute at 13,000 rpm
9. Place mini-spin in a clean 1.5 ml microcentrifuge tube
10. Add 50 µl Buffer EB or distilled water
11. Centrifuge for 1 minute at 13,000 rpm
12. For increased DNA concentrations add 30 µl elution buffer
13. Let stand for 1 minute
14. Centrifuge for 1 minute at 13,000 rpm
15. Place any material not being sequenced right away into a -80˚C freezer

Appendix E

WB2 CO1 SEQUENCE

GAGCTGGGGTAATTGGATTATCATTAAGATTAATTATTYGAATAGA
ATTAGGTTCTTGCGGTTCATTAATTGGAAATGATCAAATTTATAATTTTATT
GTTACAACTCATGCTTTTATAATAATTTTTTTTTTTGTTATACCAGTAATAA
TAGGAGGATTTGGAAATTTTTTAGTTCCTTTAATATTAGGAGCTCCTGATA
TAGTTTTTCCTCGAATAAATAATATAAGATTTTGATTATTACCTCCAAGACT
TATATTATTAATTTCAAGAATATTTGTAGGAACAGGAACAGGAACAGGAT
GAACAGTTTATCCTCCTTTATCTTTAAATGTTTCTCATGGGGGCCCTTCTGT
AGATTTATCAATTTTTTCTCTTCATATTGCAGGGGCTTCTTCAATTATAGGA
TCAATTAATTTTATTTCAACAATTTTTAATATAAAAATTTTTAAAATTGAAA
ATGTTTCTTTATTTTGTTGATCAGTATTTTTAACTGCAATTTTATTATTATTA
TCTTTACCTGTATTAGCAGGGGCTATTACAATATTATTATTTGATCGAAATT
TAAATACTTCTTTTTTTGATCCTGCAGGAGGTGG

Appendix F

COLLECTING METHODS

Family Fulgoridae

Calyptoproctus marmoratus was searched for during the spring, summer and fall of 2017-2019 by sweeping in deciduous forests and using light traps, but no individuals were obtained during the three years. Searched locations include the University of Delaware, Ashland Nature Center, C&D Canal, and Bladen County, North Carolina.

Crypoptus belfragei was searched for during the spring, summer and fall of 2017-2019 by sweeping in grass fields and undergrowth in deciduous forests and using light traps, but no individuals were obtained during the three years. Searched locations include the University of Delaware, Ashland Nature Center, C&D Canal, and Bladen County, North Carolina.

Poblicia fuliginosa was field collected in Bladen County, North Carolina, in Bladen State Forest and Jones Lake State Park during the field seasons of 2017, 2018, and 2019. All collected specimens were found on *Rhus copallinum* (winged sumac) of varying heights by inspecting from the base of the tree to 10 feet up by sight. Collection of individuals was done by hand. To capture nymphs one hand would be used as a distraction in front of the insect while the other hand would be used to capture the insect. The distraction hand would be held near the insect's dorsal side while the other hand would be on the opposite side of the trunk of the tree. Then the non-distraction hand would completely wrap around the trunk of the tree and the

insect. Nymphs would be held against the trunk and then moved into a closed fist. Hand-collected individuals were placed in a 12”x12”x12” white mesh cage with a cutting of *Rhus copallinum* for transport. Adults were collected using the same distraction technique but this time the distraction hand was placed in front of the insect roughly one foot away. The capturing hand was then slowly moved closer to the dorsal side of the insect until roughly 6 inches away. At this point the capturing hand would be thrust forward and wrapped completely around the tree. Adults were then transferred to a 12”x12”x12” white mesh cage with a cutting of *Rhus copallinum*. All collected nymphs and adults were later transferred to a larger 12”x12”x24” mesh rearing cage. Small holes were cut into water bottle lids and larger *Rhus copallinum* branches roughly 20” in length were placed through the holes cut in the lids. The bottles were filled with water and paper towels were wrapped around the base of the cutting before inserting the cuttings into the water bottle. The paper towels were used to fill the gaps between the hole and the plant cutting so no individuals would be able to descend the cutting into the water. The 12”x12”x 24” rearing cage was kept outside during the week of collecting and a tarp was suspended over it to prevent rain from coming in direct contact with the cage (reducing the impact of weather on the insects). All *Poblicia fuliginosa* collected over the course of each week were stored in this single rearing cage and then transported to the University of Delaware at the end of the collecting trip. In 2017 collections were made from September 17th to September 21st. In 2018 colletions were made from August 18th to August 23. In 2019, collections were repeated August 24th to 28th in Bladen County, NC.

Family Dictyopharidae

Rhynchomitra microrhina was searched for during the spring, summer and fall of 2018-2019. Asteraceous fields and wetland habitats with dense plant material were targeted at the University of Delaware, Ashland Nature Center, and Bladen County, North Carolina. *Aster* sp. and goldenrod were targeted during collection and yielded higher numbers of adults when swept in contrast to random sweeping of all plants. Individuals found from sweeping were collected using a size 4 Rose Entomology aspirator and brought back to the University of Delaware in the collection vial of the aspirator.

Scolops sulcipes was searched for during the spring, summer and fall of 2018-2019. Asteraceous fields were targeted at the University of Delaware, Ashland Nature Center, C&D Canal, and Bladen County, North Carolina. Individuals found from sweeping were collected using a size 4 Rose Entomology aspirator and brought back to the University of Delaware in the collection vial of the aspirator.

Family Flatidae

Flatormenis proxima, Metcalfa pruinosa, and Ormenoides venusta were searched for during the spring, summer and fall of 2017-2019 at the University of Delaware, Ashland Nature Center, and C&D Canal. Sight searching and sweeping of plant material at all locations were used for collection. *Robinia pseudoacacia* (black locust) was targeted while collecting because it is a food source and was needed for rearing. All insects were collected from the net using a size 4 Rose Entomology Aspirator and brought back to the University of Delaware in the aspirator collection tube.

Family Acanaloniidae

Acanalonia conica and *Acanalonia bivittata* were searched for during the spring, summer and fall of 2017-2019 at the University of Delaware, Ashland Nature Center, and C&D Canal. Sight searching and sweeping of plant material at all locations were used for collection. *Robinia pseudoacacia* (black locust) was targeted while collecting because it was a food source and it was used for rearing. All insects were collected from the net using a size 4 Rose Entomology Aspirator and brought back to the University of Delaware in the aspirator collection tube.

Family Issidae

Aplos simplex was searched for during the spring, summer and fall of 2017-2019 at the University of Delaware, Ashland Nature Center, and C&D Canal. Sight searching and sweeping of plant material at all locations were used for collection. *Robinia pseudoacacia* (black locust) was targeted while collecting because it was a food source and was used for rearing. All insects were collected from the net using a size 4 Rose Entomology Aspirator and brought back to the University of Delaware in the aspirator collection vials.

Family Membracidae

Thelia bimaculata was searched for during the spring, summer and fall of 2019 at the University of Delaware and the C&D Canal. Sight searching was used to locate adults on *Robinia pseudoacacia*. For areas with dense plants material, the ends of all reachable branches were placed in the bag portion of an insect net and shaken vigorously for roughly 5 seconds, dislodging any adults on the branches of the trees. All adults collected either by hand or in a net were then aspirated using a size 4 Rose Entomology Aspirator and brought back to the University of Delaware. During the

first collecting attempt roughly 20 individuals died while being transported back to the University due to heat. Subsequently, a cooler was used to transport the aspirator vials back from subsequent collection trips. No ice was used in the cooler, but it was kept in shaded areas of the car during travel. All adults survived the travel using this method.

Family Reduviidae

Arilus cristatus was not located in the field during collection attempts. Seven females and two males were instead obtained from fellow University of Delaware employees who donated them for this research. A total of 8 individuals (6 females and 2 males) were donated between September 25th to October 12th, 2019.

Pselliopus barberi individuals were all collected during the 2019 field season from the University of Delaware Lepidopteran Trail. Three individuals were collected, the first on August 21st, and the last adult on August 23rd. All three specimens were collected by sweeping goldenrod that were in direct sunlight. Two were collected as nymphs and the third was collected as an adult.

Family Coreidae

Leptglossus fulvicornis individuals were collected on *Magnolia virginiana* on the University of Delaware campus. A total of 3 fifth instar nymphs and 3 adults were collected on a single tree between August 15th and September 5th, 2019.

Anasa tristis adults and nymphs were collected during the 2019 field season at the University of Delaware. The first two adults were found in Townsend Hall on June 24th and 27th, 2019. A total of 22 nymphs of varying instars were collected on *Cucubita* sp. on July 27th outside of Townsend Hall, Newark.

Appendix G

REARING AND EGG COLLECTION METHODS

Family Fulgoridae

Collected *P. fuliginosa* during 2017 were brought back to the University of Delaware and kept in a 12”x12”x24” rearing cage in ambient laboratory conditions. They were given *Rhus copallinum* cuttings placed in water like field collection methods mentioned previously. Cuttings were replaced as wilting was observed.

Collected *P. fuliginosa* during the 2018 and 2019 field seasons were brought back to the University of Delaware and kept in a custom-built rearing cage. The cage was constructed out of 2”x2” pressure treated square wooden boards and mesh screening. The cage measured 4.5 ft long x 2.5 ft wide x 5 ft tall. The cage was kept in a greenhouse under natural light conditions with a temperature range from 22˚C to 35˚C and an average humidity of 24%. Four potted *Rhus copallinum* measuring roughly 4” tall were placed in the rearing cage for use as food. Plants were watered every-other day.

Family Dictyopharidae

During the 2018 field season collected *Rhynchomitra microrhina* were kept in the laboratory under ambient conditions in a 12”x12”x24” mesh rearing cage to allow for close observation of copulation or egg deposition. In the rearing cage three 20” potted goldenrod plants were placed in the bottom of the cage. Plants were watered every-other day, and visually inspected for eggs after all adults died.

During the 2019 field season collected *Rhynchomitra microrhina* were placed in a 24”x24”x36” rearing cage kept in a greenhouse under the same conditions stated for *Poblicia fuliginosa*. A 28 quart under bed storage container measuring 23.5” wide x 16.12” deep x 6” high was placed at the bottom of the cage. This container provided plants with more area than small pots and allowed more water to be retained, simulating a wetland environment. No holes were drilled into the container for the first attempt, but the water retention killed all goldenrod planted in it. Several holes were drilled in the container before a second planting attempt and this proved to be a more viable planting method as no goldenrod died after being planted. The container was watered every other day.

During the 2018 field season, collected *Scolops sulcipes* were kept in the laboratory under ambient conditions in a 12”x12”x24” mesh rearing cage. They were kept in the lab to allow for close observation to observe copulation or egg deposition. In the rearing cage three 20” potted goldenrod were placed at the bottom of the cage. Plants were watered every other day. After all adults died, plants were removed from the cages and visibly inspected for eggs or signs of eggs inserted into the plant. After a visual inspection of the plants, all plants were cut into 10” pieces and the main stem was sliced in half. After the plant was completely removed and inspected, the top 2” of soil was removed and placed into petri dishes. The soil was observed under a microscope to see if any eggs were deposited subsurface.

During the 2019 field season all *Scolops sulcipes* collected were placed in a 24”x24”x36” rearing cage in a greenhouse with a 28-quart container at the bottom planted with *Solidago* sp. identical to the set-up used for *Rhynchomitra microrhina*. The container was watered every other day.

Rearing of Flatidae, Acanaloniidae, Issidae, and Membracidae

All species within the families Flatidae, Acanaloniidae, Issidae, and Membracidae were reared in the same manner as described above to obtain eggs. All collected insects were then placed in a 24”x24”x36” rearing cage in a greenhouse. The greenhouse was under natural light conditions, the temperature ranged from 22˚C to 33˚C and the average humidity was 24%. Two potted 18”-24” tall *Robinia pseudoacacia* were placed in the rearing cage to provide food and as a substrate for females to deposit eggs. Plants were watered every other day and replaced once during the experiment to reduce pest infestations. During the 2017 field season, all plants were deconstructed and any eggs found were sent to USDA APHIS cooperators for host range tests. During the 2018 field season half of the plants containing eggs were moved to a lightly heated greenhouse with a temperature of 15˚C to provide a cooler period for the eggs while still keeping them in the plant material.

After all adults had died, plants were visually inspected for eggs. All plant material that fell off the plant during the field season was brought into the lab and inspected under a microscope.

Metcalfa pruinosa rearing cages included chunks of highly textured bark hung on the sides and placed against the trunks of the *Robinia pseudoacacia* in an attempt to promote egg laying. After all adults had died the plants and bark pieces were visually inspected for eggs. All plant material that fell off the plant during the field season was brought into the lab and inspected under a microscope. Bark was held over white sorting trays and tapped in an attempt to dislodge any eggs not seen during visual inspections. All debris knocked off the bark was inspected under a microscope.

Family Acanaloniidae

After all adults had died plants were visually inspected for eggs. All plant material that fell off the plant during the field season was also brought into the lab and inspected under a microscope.

Family Issidae

After all adults had died plants were visually inspected for eggs. All plant material that fell of the plant during the field season was also brought into the lab and inspected under a microscope. One plant was then taken into the lab and all branches were removed from the main trunk. All branches and the trunk were cut into 10" pieces and inspected under a microscope for eggs or signs of eggs being inserted into the plant. After all plant material was inspected, each branch and each piece of the trunk was cut in half vertically and examined under a microscope to see if there were any eggs inserted into the plant that were missed during the external inspection. All soil in each pot was also sifted and observed under a microscope for any subterranean eggs.

Family Membracidae

After all adult *Thelia bimaculata* died, plants were visually inspected for signs of eggs being inserted into the plant which was described by Funkhouser (Funkhouser 1915). After visual inspection one of the plants was taken into the lab and all branches were removed from the main trunk. All branches and the main trunk were cut into 10" pieces and observed under a microscope.

Family Reduviidae

Arilus cristatus individuals were housed separately after they were received. The first male and female received on September 25th were fed twice before introduction into a single enclosure to prevent the female from eating the male. Three containers were used for housing based on availability. The first housing method was the used of either a small Kritter Keeper (9"length x6" width x 6.5" height) or a medium critter keeper (11.7" length x7.6" width x8"height) or a one quart soup container with a large hole cut out of the lid. Fine mesh screening was placed over each container before the lid was placed on to provide a ceiling area for the insects to hold onto. Paper towels were placed flat against the bottom of the containers as a substrate and a crumpled-up paper towel was placed in each enclosure to provide a place to hide. Each adult was offered one *Achroia grisella* larvae every other day using feeding tongs. Twice a week a single adult cricket purchased at a local pet store was placed in each cage to allow for hunting. Cages were kept in ambient lab conditions near windows to supply a natural day/night cycle.

Pselliopus barberi were kept separately in one-quart soup containers with a hole cut in the lid and fine mesh screening placed under each lid. Paper towels were used as a substrate and hiding place like the rearing methods of *Arilus cristatus*. *Anasa tristis* nymphs as well as *Achroia grisella* were offered as food every other day. Cages were kept in ambient lab conditions near windows to supply a natural day/night cycle.

Family Coreidae

Leptoglossus fulvicornis individuals were kept in a 12" length x12" width x24" high metal rearing cage. Several cuttings of *Magnolia virginiana* were placed in beakers filled with water and wrapped with cotton to prevent insects from crawling into the water. All cuttings contained at least one magnolia fruit and several leaves.

The cage was kept in an ambient conditions in a laboratory near a window to provide a natural day/night cycle. Cuttings were replaced as needed.

Anasa tristis individuals were all placed in a single 12” length x12” wide x24” high rearing cage. The rearing cage was originally kept in an ambient laboratory for observation but was moved to a greenhouse for a short time before returning it to the laboratory. Several potted *Cucurbita pepo* grown from seed and measuring between 7”-10” were offered as food sources for the colony. They were watered every other day and were replaced when needed.

Appendix H

BEHAVIORAL OBSERVATIONS

Family Fulgoridae

Poblicia fuliginosa nymphs were more reluctant to abandon their plant when disturbed, compared to the adults, which would jump from their plant roughly 25% of the time when approached for collection. Nymphs would instead run around the trunk to the opposite side of the disturbance and begin to shake when closely approached. This shaking might be used to confuse predators and give the nymph enough time to jump away from the tree. Adults were found from the base of the plant on the trunk up into the branches (the highest one found was seven feet up). Rarely were adults found more than 12 inches out on a vertical branch, and this might be due to lack of food moving through the plant in outer branches compared to thicker branches closer to the trunk or the trunk itself. It could also be due to the lack of shelter from detection the father out they are on the tree.

On the first collection trip a large sandy road following a power line was walked for roughly 4,100 feet and approximately 150 trees were inspected. On a single tree 13 adults were counted, of which ten were collected. An additional inspection was carried out the following day. All trees were barren except the one tree from the day prior, which then contained eight adults. One last inspection was carried out on day 3, but the tree only contained 5 adults. During the next field season this site was revisited. All trees were vacant of *P. fuliginosa* except the “favored tree” from the year before. Nymphs and adults were found on the tree during the second year of

collecting, but numbers were less than the previous year, possibly due to collecting. The third year the area underwent clear-cutting prior to collecting and the "favored tree" had been cut down. The track was followed again and only small saplings (roughly one foot tall) were found. The saplings were roughly 1,190 feet from where the "favored tree" was located. Four of those saplings contained one nymph each.

Family Reduviidae

Arilus cristatus females spent much of their time attached to the mesh under the lids of the enclosers, and rarely were found within the paper towel refugia provided. Prey offered with forceps was often attempted while females were on the mesh roof, but most attempts resulted in the induvial attempting to flee. If food was placed in front of the insects and continuously moved it was often attacked. When triggered to attack, the individual would raise up both front legs before reaching out to grasp the prey. If prey acquisition was successful, they would quickly probe the prey with their proboscis in what appeared to be an attempt to find a suitable area for stabbing the prey. Once a suitable location was found they would quickly insert their proboscis into the prey and hold on tightly to limit the possibility of escape.

Pselliopus barberi exhibited much more aggressive behavior towards prey than *A. cristatus*, with individuals taking prey held with forceps without hesitation. If prey was placed in the enclosure and visually seen by *P. barberi* they would rush towards it and grasp the prey quickly with their front legs. They would quickly insert their proboscis and hold onto the prey. The nymphs were able to restrain and kill prey twice their body size.