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EXPLORING PREDATOR-PREY INTERACTIONS IN AGROECOSYSTEMS THROUGH MOLECULAR GUT-CONTENT ANALYSIS

DISSERTATION

A dissertation submitted in partial fulfillment of the
Requirements for the degree of Doctor of Philosophy in Entomology in
The College of Agriculture, Food and Environment
At the University of Kentucky

By
Kacie Jo Athey

Lexington Kentucky

Director: Dr. John J. Obrycki, Professor of Entomology

Lexington Kentucky

2017

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ABSTRACT OF DISSERTATION

EXPLORING PREDATOR-PREY INTERACTIONS IN AGROECOSYSTEMS THROUGH MOLECULAR GUT-CONTENT ANALYSIS

Generalist predators can contribute to vital ecosystem services by potentially inducing trophic cascades as natural enemies of pests in agroecosystems. As the human population of the world gets larger, we need to produce more food on ever-smaller swaths of available land relying on ecosystem services, in the form of pest control, that may contribute to agricultural sustainability. Teasing apart the exact trophic linkages between predators and prey is a vital first step and essential to uncovering which predators are inducing trophic cascades and should be enhanced through conservation biological control.

Combined with ecological experimentation, the main tool used throughout my research to identify trophic linkages is molecular gut-content analysis. I began by investigating mass sampling techniques and found they do not cause contamination in gut-content analysis and may be a simple method for collecting large numbers of cryptic predators for use in determining trophic linkages. Additionally, my research uncovered trophic interactions between stink bugs and generalist predators at multiple scales. Overall, I successfully designed molecular methods to investigate relationships between agricultural pests and generalist predators. A multi-year field study uncovered low predation on stink bug pests in contrast to previous research suggesting that generalist predators were contributing highly to biological control. This research highlights the need for replicated studies before making broad conservation biological control decisions. Although generalist predators were not consuming stink bugs in large numbers, my field cage study showed evidence of superfluous killing by spiders on adult stink bugs, highlighting the need to combine ecological studies with molecular methods to understand consumptive and non-consumptive effects on prey items. Gut-content analysis showed no evidence of consumption, but the field cage study allowed me to uncover the complicated relationships between spiders and stink bugs. In addition, I showed an invasive species can be detected in new areas through molecular gut-content analysis of predators before other sampling methods.

KEYWORDS: Agroecosystems, Pentatomidae, Food Webs, Generalist Predators

Kacie Jo Athey

April 26, 2017
Date

EXPLORING PREDATOR PREY INTERACTIONS IN AGROECOSYSTEMS THROUGH MOLECULAR GUT-CONTENT ANALYSIS

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Chapter 1: Introduction

1.1 Role of generalist predators in trophic cascades in agroecosystems

Predator prey interactions are affected by many abiotic and biotic factors. In agricultural systems, the interplay between weather, habitat structures, pesticides, harvest cycle, non-crop vegetation, pests, natural enemies and other arthropods combine to form complex interaction webs of organisms (Welch and Harwood 2014). The species in these webs can have both direct and indirect effects on each other and other organisms in their community (Eubanks and Finke 2014, Welch and Harwood 2014).

Predators can indirectly affect lower trophic levels through trophic cascades (Paine 1980, Carpenter et al. 1985) occurring when predators consume prey (e.g., herbivores), thus indirectly benefiting the resource base of their prey (e.g., plants as primary producers). Generally, trophic cascades occur when there are at least two links in a trophic chain and reduction in abundance or biomass at one level results in an increase in abundance or biomass at a different level. For example, when a predator reduces the abundance of a prey item or alters its behavior and the next trophic level down, a plant, increases in biomass (Beckerman et al. 1997, Schmitz and Suttle 2001). In a four-level system, a top predator could prey on a mesopredator and cause an herbivore to increase in abundance thereby decreasing the abundance of the plant the herbivore is feeding on (Whitehouse et al. 2011). In a multiple predator system, several species may experience cascading effects at lower trophic levels corresponding with the most prolific predator in the system (Nyström et al. 2001). These cascades are common (Pace et al. 1999, Schmitz et al. 2000, Halaj and Wise 2001, Shurin et al. 2002, Estes et al. 2011) and modified by

many factors such as habitat type, food quality, intraguild predation (Hatcher et al. 2006) and predator-prey body size ratios (Shurin et al. 2002, Grabowski 2004, Borer et al. 2005, Preisser et al. 2005, Shurin et al. 2006).

In agroecosystems, trophic cascades caused by biological control are the goal of interactions between generalist predators and herbivorous pests and have been demonstrated with various predator taxa (Riechert and Bishop 1990, Carter and Rypstra 1995, Snyder and Wise 1999, Colfer and Rosenheim 2001, Snyder and Wise 2001, Halaj and Wise 2002, Cardinale et al. 2003, Rypstra and Marshall 2005, Finke and Denno 2006, Costamagna et al. 2007). In most cases, more than one predator contributed to these trophic cascades. Groups of predators, in general, are more effective at controlling prey than a single predator species (Chiverton 1987, Riechert and Lawrence 1997, Cardinale et al. 2006, Letourneau et al. 2009). This control occurs when positive interactions among natural enemies, such as resource partitioning, are stronger than negative interactions, such as intraguild predation (Letourneau et al. 2009). Positive interactions can include, facilitation of prey capture for another predator (e.g., aphid dropping behavior elicited by a foliar predator resulting in consumption of aphid by a ground predator (Losey and Denno 1998)), resource partitioning among natural enemies increasing overall pest control (Finke and Snyder 2008), and overall higher pest suppression (Northfield et al. 2014).

Generalist predators may be ideal candidates for inducing trophic cascades through biological control as they can maintain high population levels in agroecosystems by exploiting non-pest prey when specialists cannot (Symondson et al. 2002, Welch et al. 2012, Welch and Harwood 2014). Generalist predators can maintain populations in

agricultural fields, subsisting on alternative prey resources, until the pests first arrive, thereby having a greater overall impact on the pest population, resulting in trophic cascades with net benefits for plants (Welch and Harwood 2014, Athey et al. 2016).

Trophic cascades can result in benefits that people obtain from ecosystems, referred to as ecosystem services (MEA 2005). Generalist predators contribute to vital ecosystem services by inducing trophic cascades as natural enemies of pests. These ecosystem services, though important, are often sacrificed through agricultural intensification (Tilman et al. 2002, Foresight 2011). As the human population of the world gets larger and larger, we are tasked with producing more food on ever-smaller swaths of available land (MEA 2005). We need to utilize sustainable farming practices where high yields can be maintained, farms can withstand major change and have acceptable environmental impacts (Conway 1997). Relying on ecosystem services, in the form of pest control, may pave the way for more sustainable agricultural practices. Research has shown biological control is an important ecosystem service in agroecosystems and can aid in promoting and implementing sustainable agricultural practices (Landis et al. 2000, Losey and Vaughan 2006, Isaacs et al. 2009, Ragsdale et al. 2011).

Conservation biological control can enable predator populations to be enhanced through management of the local flora and fauna. Habitat manipulation enables the enhancement of natural enemy populations through provisioning of resources typically lacking in agricultural fields (Landis et al. 2000), and can strengthen top-down control of insect pests (Costamagna et al. 2007, Holland et al. 2012). These provisions include extra-floral nectaries (Edwards et al. 1979, Baggen and Gurr 1998, Heil 2015), refugia

for predators (Halaj 2000, Knapp and Rezac 2015), and alternative prey (Gurr et al. 2004, Landis et al. 2005). For example, field margins provide alternative prey for natural enemies (Thomas and Marshall 1999, Frank et al. 2009, Bickerton and Hamilton 2012), act as a trap for pests (Deol and Rataul 1978, Fereres 2000, Mitchell et al. 2000, Hooks and Fereres 2006), and may ultimately reduce damage to the crop (Balzan and Moonen 2013). In addition, providing refugia increases spider abundance, spider species richness in soybeans increased by 60% with a concomitant 33% reduction in damage to seedlings in manipulated plots (Halaj 2000). Moreover, in cropping systems with aphid pests, strip-planting with ryegrass may replace at least one insecticide application per season, which has long-term economic and environmental benefits (Dong et al. 2012). Studies of multiple predators on pest species are important to help understand what role enemy biodiversity plays in biological control and if it can influence trophic cascades (Costamagna et al. 2007). Teasing apart the exact trophic linkages between predators and prey is essential to uncover which predators are inducing trophic cascades and should be enhanced through conservation biological control and is a vital first step. The main tool used throughout this dissertation to identify trophic linkages is molecular gut-content analysis.

1.2 Molecular gut-content analysis

1.2.1 Uses for molecular gut-content analysis

One of the major advances in food web ecology and biological control is the use of molecular tools to verify trophic connections within hypothesized interaction webs. Molecular gut-content analysis is useful for screening diverse predators in a short amount of time for a given prey item. For vertebrates, visually screening feces can reveal trophic

linkages but the majority (≥ 79%) of terrestrial arthropod predators feed by liquid ingestion following extra-oral digestion (Cohen 1995), with visual inspection of gut-contents revealing nothing other than if the predator had eaten recently.

The detection of prey-associated molecules such as proteins (enzyme-linked immunosorbent assay (ELISA)) or DNA (polymerase chain reaction (PCR)) in the guts of predators provides insight into trophic connections structuring food webs (Sheppard and Harwood 2005, Juen and Traugott 2007, King et al. 2008, Weber and Lundgren 2009, Hagler 2011, Hagler and Blackmer 2013, Furlong 2015, Hagler et al. 2015). Molecular gut-content analysis employing PCR is a popular tool for characterizing trophic linkages across a variety of habitats, including vegetables (Balmer et al. 2013, Schmidt et al. 2014), row crops (Agustí et al. 2003, Hagler and Blackmer 2013, Lundgren and Fergen 2014), forage crops (Welch et al. 2014), fruit (Boreau de Roincé et al. 2013, Mollot et al. 2014)), forest systems (Schoeller et al. 2012, Heidemann et al. 2014, Jelaska et al. 2014), and soil (Lundgren and Fergen 2014, Wallinger et al. 2014).

1.2.2 Limitations to molecular gut-content analysis

As with any technique, there are limitations to estimating consumption patterns with molecular gut-content analysis. For example, if decay rates of gut-contents are high, predation can be under-estimated as there is a very short detection window (Greenstone et al. 2014b). Variables such as predator identity, prey identity, sex, and temperature influence the rates of decay and the detection window size (Hagler and Naranjo 1997, Greenstone et al. 2014b), making it difficult to determine the predation rates. In addition, scavenging and secondary predation (consumption of another predator that had consumed the target prey itself) cannot be separated from primary predation and may inflate trophic

linkage estimates. For instance, von Berg et al. (2012) found that 26-41% of predator species tested consumed dead aphids when offered both live and dead aphids. Similarly, cannibalism cannot be detected using PCR as this method cannot distinguish between individuals (Gagnon et al. 2011). Additionally, molecular gut-content analysis is only a qualitative measure of predation unless controlled feeding trials and statistical analyses using Bayesian or other non-parametric methods are used in tandem to determine relative predation levels (Greenstone et al. 2010, Welch et al. 2014).

Undetected contamination could also lead to higher gut-content positives. A major advantage of molecular gut-content analysis utilizing specific primers is their sensitivity; well-designed primers can detect trace amounts of prey DNA. However, the sensitivity of polymerase chain reaction (PCR) makes it susceptible to contamination by minute concentrations of DNA from various sources (Sheppard and Harwood 2005, King et al. 2008). Several studies have attempted to quantify this contamination (Chapman et al. 2010, Greenstone et al. 2011, Greenstone et al. 2012, King et al. 2012, O'Rorke et al. 2013). For example, Greenstone et al. (2011) explored whether a rough collecting method, beat sheeting followed by mass collecting via an aspirator, would cause gut contamination. The authors found that 31% of the predators in the rough treatment had cross-contamination. In contrast, Chapman et al. (2010) tested vacuum sampling for molecular gut-content analysis and found no evidence of cross contamination. With various techniques having opposite outcomes, contamination during the sampling and storage process needs further testing and in Chapter 2, I investigated possible contamination resulting from a common collection method (pitfall traps) and mass storage of specimens destined for molecular gut-content analysis.

Molecular gut-content analysis is also incapable of uncovering non-consumptive effects and these effects have been well documented to alter pest behavior, survival, and reproduction. For instance, superfluous killing (Maupin and Riechert 2001), could result in mortality for a prey item without consumption by the predator. Many groups of animals, including tardigrades, (Hohberg and Traunspurger 2009), mammals, (Short et al. 2002), insects (Johnson et al. 1975, Lang and Gsodl 2003, Lounibos et al. 2008) and spiders (Riechert and Maupin 1998, Maupin and Riechert 2001, Trubl et al. 2011) exhibit this behavior. Predator-induced behavioral changes (decreased feeding rates and reproduction) can also negatively affect prey populations without consumption by the predator (Schmitz et al. 1997, Brown et al. 1999, Preisser et al. 2005, Preisser and Bolnick 2008, Sitvarin et al. 2016). In Chapter 3, I investigated possible non-consumptive and consumptive effects between a generalist predator and agricultural pest by combining a field cage study with molecular gut-content analysis.

Despite its limitations, molecular gut-content analysis is a useful tool for visualizing food web linkages especially when coupled with pest and predator abundance data (Furlong 2015). Although these approaches allow reliable post-mortem identification of prey items contained in the gut of the predator, they do not alone quantify biological control. Combining molecular gut-content analysis with ecological experimentation can provide a more complete understanding of predator-prey interactions. In this dissertation, I combined molecular gut-content analysis with a field cage experiment (Chapter 3) and a two-year field study across three locations in two crops (Chapters 4 and 5).

1.3 Stink bugs and relatives as pests in agroecosystems

Much of this dissertation (Chapters 3-5) focuses on combining ecological experimentation and molecular gut-content analysis to investigate predator effects on stink bugs and kudzu bugs in ecosystems of economic importance. Stink bugs (Hemiptera: Pentatomidae) are significant pests in soybean (Turnipseed and Kogan 1976, Panizzi and Slansky 1985) and cotton (Greene et al. 2001, Williams 2013) in the United States. Stink bugs are not affected by Bt (*Bacillus thuringiensis* Berliner) (Bacillales: Bacillaceae) containing plants or sprays. The widespread adoption of Bt cotton and the boll weevil eradication program has led to decreased broad-spectrum insecticide use in the southern United States (Ruberson et al. 2012) and allowed stink bugs to become significant pests in crop systems (Turnipseed et al. 1995, Greene et al. 1999).

Stink bugs are a monophyletic, cosmopolitan group of phytophagous and predaceous species (McPherson and McPherson 2000). Stink bugs emit a foul smell from their dorsal abdominal glands when disturbed. It has been suggested that one of the main functions of these secretions is predator avoidance (Pavis et al. 1994). Aggregation is common in all life stages and both sexes (Inkley 2012, Reay-Jones 2014), in response to pheromones released by adult males, likely for resource identification (Weber et al. 2014). Second through fifth instar nymphs are highly responsive to these pheromones and will aggregate in response to them (Khrimian et al. 2014, Lee et al. 2014, Leskey et al. 2015). First instar nymphs produce unique secretions, remain together on the egg mass until they molt, and do not feed (Simmons and Yeargan 1988, Todd 1989, Borges and Aldrich 1992, McPherson and McPherson 2000). Many members of Pentatomoidea are agricultural pests; the most economically important in North America being the southern

green stink bug (*Nezara viridula* (L.)); the green stink bug (*Chinavia hilaris* (Say)); brown stink bug (*Euschistus servus* (Say)); rice stink bug (*Oebalus pugnax* (Fab.)); one-spotted stink bug (*Euschistus variolarius* (Palisot de Beauvois)); the brown marmorated stink bug (*Halyomorpha halys* (Stål)), and the kudzu bug (*Megacopta cribraria* (F.)). The focus of research in this dissertation were trophic linkages between generalist predators and *H. halys* (Chapter 3); *N. viridula, C. hilaris*, and *E. servus* (Chapter 4); and *M. cribraria* (Chapter 5).

1.3.1 Brown Marmorated Stink Bug

The brown marmorated stink bug (*Halyomorpha halys* (Stål)) is an invasive species native to China, Korea and Japan, that was accidentally introduced into the United States in 1996 in Allentown, PA (Hoebeke and Carter 2003). It is a pest of many important crops as well as a household nuisance because it tends to enter homes to overwinter (Hoebeke and Carter 2003). Since its entrance into the United States, *H. halys* has spread quickly and is currently found in 43 states (StopBMSB 2017). As of January 2017, *H. halys* is considered a severe agricultural and nuisance pest in nine states, and a nuisance and agricultural pest in an additional ten states (StopBMSB 2017). *Halyomorpha halys* is a strong flier and will easily hitch a ride on vehicles aiding its spread immensely (UMD Entomology, 2010).

A female can lay eggs for nearly half her life span when she has only mated once. Fecundity decreases in relation to age, but with multiple matings, fecundity and the period of egg laying increases (Hoebeke and Carter 2003). In the greenhouse, egg masses with a median of 28 eggs are laid on the underside of leaves, with a mean lifetime total of

212 eggs (Nielsen et al. 2008). *Halyomorpha halys* are univoltine through most of their native range (Zhang 1993). In a survey near Allentown, PA, *H. halys* was the dominant stink bug species on the plants sampled (Nielsen and Hamilton 2009). *H. halys* is also not innocuous in its native range and is a pest on several crops including peach (*Prunus persica* (L.) Batsch), cherry (*Prunus* spp.), apple (*Malus pumila* Miller), plum (*Prunus* spp.), fig (*Ficus carica* L.), persimmon (*Diosphyros kaki* L.f.), orange (*Citrus* x *sinensis* (L.) Osbeck), grape (*Vitis* spp.), mulberry (*Morus* spp.), and soybean (Kobayashi 1967, Funayama 2004, UMD Entomology 2010).

1.3.2 Southern Green Stink Bug

Southern green stink bug, *Nezara viridula*, (L.), most likely a native of Ethiopia (Kavar et al. 2006), has a cosmopolitan distribution (Todd 1989) and is one of the most important stink bug pests in soybean in the southern United States (McPherson et al. 1979, McPherson and McPherson 2000). *Nezara viridula* is polyphagous, attacking over 30 species of plants (Todd 1989, Panizzi 2000, Panizzi et al. 2000). They have three to four generations per year in temperate climates (Smith et al. 1986) and overwinter in protected areas, such as under litter or bark (Jones and Sullivan 1981). Overwintering adults are active and feed, greatly enhancing their survival (Todd 1989). *Nezara viridula* prefers to feed on plants during fruit formation (McPherson and McPherson 2000) and development from egg to adult takes about 35 days in summer, depending on temperature (Todd 1989).

1.3.3 Green Stink Bug

Green stink bug, *Chinavia hilaris* (Say) is native to North and South America. *Chinavia hilaris* overwinters as an adult in leaf litter in deciduous woods (Jones and

Sullivan 1981, Javahery 1990), is univoltine (Javahery 1990, McPherson and McPherson 2000), or bivoltine under favorable climate conditions (Wilde 1969, Jones and Sullivan 1981, McPherson and Tecic 1997). In the field, female *C. hilaris* lays 14-56 eggs per cluster with an average fecundity of 134 eggs in her lifetime (Javahery 1990) and can consume many host plants but prefers woody plants over herbaceous annuals (Jones and Sullivan 1982). Per Jones and Sullivan (1982), *C. hilaris* can utilize up to 16 different host plants including, *Photinia* sp., American holly (*Ilex opaca* Aiton), Chinese privet (*Ligustrum sinense* Lour.), trumpet-creeper (*Campsis radicans* Seem.), black cherry (*Prunus serotina* Ehrh.), elderberry (*Sambucus* spp.), peach and soybean.

1.3.4 Brown Stink Bug

Brown stink bug, *Euschistus servus* (Say), is polyphagous and prefer to feed on plants during fruit formation. *Euschistus servus* can affect the yield of soybean, wheat (*Triticum* spp.), alfalfa (*Medicago sativa* L.), corn (*Zea mays* L.), tomato (*Solanum lycopersicum* L.), peach, pear (*Pyrus* spp.), apple and pecan (*Carya illinoinensis* (Wangenh.) K.Koch) (McPherson 1982). Early in the season, adults have been found on crimson clover (*Trifolium incarnatum* L.), vetch (*Vicia* spp.) wheat, sowthistle (*Sonchus oleraceous* L.) and peppergrass (*Lepidium virginicum* L.) (Jones and Sullivan 1982). Adults overwinter in protected shelters such as leaf litter and crop residue with two generations per year in the United States (McPherson and McPherson 2000).

1.3.5 Kudzu Bug

Kudzu bug (*Megacopta cribraria* (F.)) (Hemiptera: Plataspidae) is native to Asia and was discovered in the United States in 2009 in northeast Georgia (Eger et al. 2010). It is the first member of the family Plataspidae in North America and was reported from

several locations in Georgia and found in large numbers on houses near fields of kudzu, *Pueraria montana* (Lour.) Merr. (Fabales: Fabaceae). *Megacopta cribraria* develops on kudzu and moves to houses to overwinter. Large numbers of *M. cribraria* were also found on vehicles in the area which could be a potential avenue of spread (Eger et al. 2010). *Megacopta cribraria* can withstand wind speeds of 100 km/h if attached to cloth and 40 km/h if attached to glass suggesting that they could easily attach themselves to vehicles aiding in range expansion (Takano and Takasu 2016). In seven years, *M. cribraria* has spread to 13 states (KudzuBug 2017).

Megacopta cribraria has been reported on a variety of plants, including cotton (Srinivasaperumal et al. 1992) but various experiments have confirmed relatively few primary reproductive hosts: kudzu, soybean, pigeon pea (*Cajanus cajan* L.), black eye pea (*Vigna sinensis* L.), lima bean (*Phaseolus lunatas* L.) and pinto bean (*Phaseolus vulgaris* L.) in the southeastern United States (Zhang et al. 2012, Del Pozo-Valdivia and Reisig 2013, Medal et al. 2013, Ruberson et al. 2013). In greenhouse choice tests *M. cribraria* lay the majority of eggs on soybean and kudzu (Medal et al. 2016). In no choice greenhouse experiements, females deposited eggs in soybeans in masses with an average of 18 eggs and development time is 45 -50 days (Del Pozo-Valdivia and Reisig 2013). *Megacopta cribraria* affects the growth of kudzu, one of the most serious invasive weeds in the southeast United States (Myers and Bazely 2003, Forseth and Innis 2004), causing a 33% decrease in kudzu growth in controlled field plots (Zhang et al. 2012). *M. cribraria* also removed 80% of the kudzu aboveground biomass over a period of three years in open field observations (Gardner and Olson 2016). *Megacopta cribraria* feeding

can also significantly reduce soybean yield (Seiter et al. 2013), making this insect both a potential biological control agent and a pest.

Megacopta cribraria also may have a specialized bacterial symbiont that allows them to be pestiferous. Initially investigated in another *Megacopta* species, *M. punctatissima,* when the symbiont capsule was heat treated, nymphs had developmental delays, abnormal coloring, and abnormal body shape (Fukatsu and Hosokawa 2002). Several species within the family Plataspidae produce symbiont capsules that the female lays under the egg mass. The hatchlings acquire the symbiont orally following eclosion. When deprived of the symbiont adult emergence was reduced, with 50% of nymphs dying during development in both *M. cribraria* and *M. punctatissima* (Hosokawa et al. 2006). This symbiont may also confer pest status to *M. cribraria* allowing it to exploit soybean (Brown et al. 2014). In their native range, *M. punctatissima* is a pest in soybeans and contains the same symbiont as *M. cribraria* contains in the United States. In the native range, *M. cribraria* does not contain this symbiont and it is not a pest (Brown et al. 2014).

1.4 Objectives

The overall objective for this dissertation was to elucidate predator prey interactions in selected agroecosystems using molecular gut-content analysis. Molecular gut-content analysis is a powerful tool for detecting trophic interactions between cryptically feeding predators and their prey. One of the drawbacks to this method is its susceptibility to false positives because of contamination. My first experiment explored sampling and storage techniques that had been reported to cause contamination, but were

not previously tested (Chapter 2). Specifically, I tested whether fluid-filled pitfall traps would cause gut contamination in predators that were collected within them and if storing two specimens together in ethanol would cause surface contamination.

For the second objective, I combined a field cage experiment with molecular gut-content analysis to explore predator impacts on an invasive stink bug species in soybeans (Chapter 3). In this study, I examined if non-consumptive effects, such as superfluous killing, and/or consumptive effects reduced stink bug densities.

My third objective documented trophic linkages between stink bug pests and generalist predators in soybean and cotton fields (Chapter 4). I identified predators that ate stink bugs in cotton and soybean agroecosystems. This was a two-year experiment in three different locations and represents a multi-year exploration of stink bug trophic connections using molecular gut-content analysis.

The fourth objective was to assess predation of a newly invasive pest, kudzu bug in soybean crops. I determined the impact of generalist predators on kudzu bug in open field conditions (Chapter 5). In addition, I determined that kudzu bug could be detected using molecular gut-content analysis in areas where traditional sampling methods, such as sweep sampling and visual surveys, had not yet detected this pest. This proof of concept provides the basis for molecular tools to be used for invasive species monitoring in terrestrial ecosystems.

Chapter 2: Investigating cross contamination of liquid storage methods in molecular gut-content analysis

Chapter contents published in Athey, K.J., Chapman, E.G., Harwood, J.D. 2017. A tale of two fluids: does storing specimens together in liquid preservative cause DNA cross-contamination in molecular gut-content studies? *Entomologia Experimentalis et Applicata.*

2.1 Summary

The study of food webs and trophic interactions increasingly relies on PCR-based molecular gut-content analysis. However, this technique may be prone to error from contamination of minute quantities of DNA; i.e., simply storing specimens together in a liquid medium may lead to cross-contamination. In this study, we used PCR to determine the contamination rate when (1) specimens were stored together in 95% ethanol for various time periods, and (2) predators fall into ethylene glycol-filled pitfall traps where the dying predator may inadvertently consume prey DNA-contaminated liquid. We designed experiments and PCR primers to quantify the risk of contamination for both situations and found no contamination by storing specimens together in 95% ethanol. Furthermore, zero predators contained prey DNA in their gut-contents from imbibing prey DNA-contaminated ethylene glycol. These results support the use of mass sampling techniques, like wet pitfall traps, for molecular gut-content analysis.

2.2 Introduction

Molecular-based gut-content analyses are now widely used to study food webs (e.g., Hagler and Blackmer 2013, Jelaska et al. 2014, Lundgren and Fergen 2014, Raso et al. 2014, Schmidt et al. 2014). These techniques detect trophic interactions and facilitate the

screening of hundreds or thousands of specimens in a short period of time. Furthermore, the majority (>79%) of terrestrial arthropod predators feed by liquid ingestion following extra-oral digestion (Cohen 1995), thereby rendering visual inspection of the gut-contents pointless. The major advantage of these techniques lies in their sensitivity; well-designed primers can detect trace amounts of prey DNA. However, the sensitivity of polymerase chain reaction (PCR) makes it susceptible to contamination by minute concentrations of DNA from various sources (Sheppard and Harwood 2005, King et al. 2008), leading to a number of studies that attempt to quantify the significance of such contamination (Greenstone et al. 2011, Greenstone et al. 2012, King et al. 2012, O'Rorke et al. 2013).

One source of contamination may be the insect specimen storage liquid. Shokralla et al. (2010) demonstrated that DNA from insect specimens stored in 95% ethanol contaminated the ethanol after a short period of time. They isolated and sequenced DNA from samples of ethanol in which individual insects and plant clippings were stored for 24 h, obtaining mitochondrial, nuclear, and chloroplast gene sequences matching those of their respective specimens. In cases where many specimens are placed in the same collection vial in the field, such as mass collecting with an aspirator or malaise traps, the possibility exists that contamination could falsely inflate DNA-based measurements of food web connections. These concerns led King et al. (2008) to recommend collecting predators for DNA testing into individual tubes in the field as a best practice, but the possibility of contamination was never directly tested.

Passive mass-sampling techniques, such as pitfall traps and Malaise traps, collect specimens in a manner whereby cross-species mixing in preservative is unavoidable. The study by Shokralla et al. (2010) highlights concerns regarding the validity of using these

traps for molecular food web reconstruction. Pitfall traps filled with ethylene glycol to preserve DNA and collect predator and prey specimens (Leal-Klevezas et al. 2000, Rubink et al. 2003, Vink et al. 2005) have been used to study the food habits of ground predators (Lundgren et al. 2009). It is possible that a prey falling into a pitfall trap may contaminate the ethylene glycol with its DNA. If a predator subsequently falls into the trap, it could imbibe contaminated ethylene glycol or, if whole-body extractions of predators are undertaken, surface-contaminate the specimens. It has been recommended that when using pitfall traps where the specimens are screened for molecular gut-content analysis, dry pitfall traps be used to decrease the potential risk of contamination. These traps must be checked very frequently and predators may consume other organisms that have fallen into the pitfall trap before the researcher can empty it, thereby leading to false positives (King et al. 2012). With wet pitfall traps, the risk of consumption within the pitfall trap is much lower, as the ethylene glycol kills arthropods quickly. Still, the recommended best practice is to use dry pitfall traps to avoid the risk of cross-contamination, although this has not been directly tested (King et al. 2008).

To assess the likelihood that DNA-contamination of preservative fluids leads to false-positive trophic connections when predators are screened by molecular gut-content analysis, we designed two experiments. The first experiment tested the hypothesis that storing predator and prey specimens together in ethanol would cause surface contamination, thus resulting in false-positive molecular ‘gut-content’ detection. The second experiment tested the hypothesis that predators collected from pitfall traps filled with ethylene glycol and subsequently gut-dissected would test positive for prey items that were found concurrently in the pitfall trap, without predation having occurred.

Upholding either hypothesis would have important implications for molecular gut-content analysis.

2.3 Materials and methods

2.3.1 Surface contamination

Specimens of parasitoid wasps [*Campoletis* spec. (Hymenoptera: Ichneumonidae)] and fruit flies [*Drosophila melanogaster* Meigen (Diptera: Drosophilidae)] were reared in separate laboratories in the Department of Entomology at the University of Kentucky (Lexington, KY, USA). Wasps were reared on *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) as previously described (Krell et al. 1982). Fruit flies were reared at room temperature. These taxa were chosen because we had prior knowledge of their diet and knew they could not have consumed each other, eliminating the possibility of gut-content amplification.

To quantify the likelihood of false-positive detection due to surface contamination caused by storing specimens together in ethanol, we stored one freshly frozen wasp and one freshly frozen fruit fly in an autoclaved 1.5-ml microcentrifuge tube filled with 95% ethanol at -20 °C for 1, 3, 5, 8, 10, 15, 20, 30, 45, 60, 90, or 120 days (n = 20 per time period). After the allotted time, specimens were separated with forceps into individual, autoclaved 1.5 ml microcentrifuge tubes filled with 95% ethanol and stored at -20 °C until DNA extraction.

2.3.2 Gut contamination

Slugs [*Megapallifera wetherbyi* (Binney) and *Philomycus* spp. (Gastropoda: Philomycidae)] were hand-collected from Berea College Experimental Forest (Berea, KY, USA; 37.5717°N, 84.2187°W). Live ground beetles [*Harpalus* spp. (Coleoptera:

Carabidae)] were collected by black light and pitfall trapping at the University of Kentucky Spindletop Research Farm, Lexington (38.1300°N, 84.5080°W). Beetles were maintained in an environmental chamber under controlled conditions (24 °C, L16:D8 photoperiod).

To quantify false positives due to gut contamination following collection into pitfall traps, slugs and ground beetles were placed in ethylene glycol and stored together for 0, 2, 4, 8, 12, or 24 h. We chose these taxa because both are very abundant, slugs are a prey item for ground beetles (Harper et al. 2005, Hatteland et al. 2011), and slugs exude large quantities of mucus that could lead to contamination. To determine whether the amount of preservative affected the rates of contamination, we added 50, 100, or 150 ml of ethylene glycol to a 250-ml container. A logistic regression (SAS Institute, Cary, NC, USA) was performed to determine whether volume had an effect on detection of target DNA. It did not (Wald $\chi^2 = 2.029$, $Pr > \chi^2 = 0.36$), so samples were pooled across volumes, giving n = 3 for each time period. One live slug was placed in each container and stored at 24 °C for 24 h, after which live ground beetles (starved ≥5 days) were added to each container. Multiple beetles (n = 20) were added to each container to simulate the most extreme field conditions for a pitfall trap left overnight (Winder et al. 2001). At 0 (at death), 2, 4, 8, 12, and 24 h, all beetles were removed from one container of each volume. The beetles were then surface cleaned with washes of deionized water and ethanol, transferred into individual 1.5-ml microcentrifuge tubes containing 95% ethanol, and stored at -20 °C.

Additionally, to test whether slug DNA could be amplified directly from the ethylene glycol after 24 h exposure, we sampled and extracted ethylene glycol from slug

containers prior to the addition of carabids. As each slug was removed from the jar, a small amount of ethylene glycol dripped off of the body. We refer to these as 'slug adjacent samples'. From these slug adjacent samples we collected subsamples of 2 and 10 μl. Once all specimens had been removed from the containers, three ethylene glycol samples of differing volumes (5, 10, and 20 μl) were collected. These we refer to as 'slug container samples'. Different volumes of ethylene glycol were collected to ensure sufficient liquid was available for use in DNA extraction and thereby allowing for the amplification of DNA, if present.

2.3.3 Sequencing for primer design

To obtain sequences for primer design and generate total DNA for detecting contamination, DNA was extracted using DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA, USA) following standard animal tissue protocols. Wasps and fruit flies were whole-body extracted for primer design and contamination testing. Ground beetle midguts were removed with sterilized forceps and dissecting scissors, and extracted for contamination testing. Negative-control ground beetles, which had been deprived of food for 48 h, were included to make sure the dissection did not cross-contaminate (n = 5). Approximately 0.02 g of slug tissue was extracted for primer design and contamination testing. Differing volumes of ethylene glycol (2, 5, 10, and 20 μl) were extracted for contamination testing. One negative control extraction was performed with beetles to make sure that extraction did not cross-contaminate.

DNA for primer design was amplified using general COI primers LCO-1490 (Folmer et al. 1994) and HCO-700ME (Breton et al. 2006). PCRs (25 μl) consisted of 1× Takara buffer (Takara Bio, Shiga, Japan), 0.2 mM of each dNTP, 0.2 mM of each primer,

1.25 U Takara *Ex Taq*, and template DNA (1 µl of total DNA). PCRs were carried out in Bio-Rad PTC-200 and C1000 thermal cyclers (Bio-Rad Laboratories, Hercules, CA, USA). The PCR cycling protocol was 94 °C for 1 min followed by 35 cycles of 94 °C for 50 s, 45 °C for 45 s, 72 °C for 45 s, and a final extension of 72 °C for 5 min. PCRs included a positive and a negative control. The negative control consisted of all reagents without the addition of DNA. Reaction success was determined by electrophoresis of 10 µl of PCR product in 2% SeaKem agarose (Lonza, Rockland, ME, USA) pre-stained with GelRed nucleic acid gel stain (1×; Biotium, Hayward, CA, USA). DNA sequencing was carried out at the Advanced Genomics Technologies Center (University of Kentucky, Lexington). Sequences were submitted to GenBank (accession numbers JN544697-JN544700).

Species identifications were confirmed by comparing sequences from our specimens to those available through the Identification Engine at the Barcode of Life Database (BOLD; (Ratnasingham and Hebert 2007)). Significant matches to all species were found except for *Campoletis* spec., which was identified by an ichneumonid taxonomist as a currently undescribed species (David Wahl, American Entomological Institute, pers. comm.).

2.3.4 Primer design

To design wasp and fruit fly primers, we constructed a matrix containing sequences from each species. Using *Primer3* (Rozen and Skaletsky 2000), we designed wasp-specific primers (Camp-360-F and Camp-567-R) and fruit fly-specific primers (Dros-18-F and Dros-237-R) (Table 2-1). All reaction conditions were identical to the COI protocol, except the cycling conditions were 94 °C for 1 min, followed by 35 cycles of 94

°C for 1 min, 60 °C (wasp primers) or 61 °C (fruit fly primers) for 45 s, and 72 °C for 30 s. Primers were designed only to distinguish these two taxa and were not tested for cross-amplification with other species. To design slug primers, we generated a matrix of COI sequences. Slug-specific primers (Phylo-32-F and Phylo-332-R; Table 1) only differentiated slugs from the focal ground beetles. Reaction conditions and cycling protocol were identical to the wasp protocol. All samples of ethylene glycol were screened with these primers to determine whether the storage liquid was directly contaminated.

Primer sensitivity for all primer pairs was determined by testing dilutions of target DNA for amplification. DNA concentration from the original extractions was determined using a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany) adjusted to 5 000 pg μl^{-1} and two-fold serially diluted. The serially diluted target DNA was used as a template for each primer pair at concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, 0.05, and 0.025 pg μl^{-1} of target DNA. Finally, to confirm extraction success, all ground beetles were tested with the general COI primers, and all wasp and fruit fly extractions were tested with the primers designed specifically to amplify their respective taxa.

2.4 Results

2.4.1 Primer functionality

All primer pairs amplified 100% of targets and 0% of non-targets. All primers designed here are specific to this project and may not be of utility outside of this limited scope without extensive non-target testing for which they were not optimized. The

sensitivity of the primer pairs varied with the lowest concentration amplifiable listed in Table 2-1 ranging from 0.05 pg μl^{-1} for the slug primers to 6.25 pg μl^{-1} for the fruit fly primers.

2.4.2 Surface contamination

A total of 238 wasps were tested for fruit fly DNA (two extractions failed and were discarded), and zero tested positive for fruit fly DNA. In total 239 fruit flies were tested for wasp DNA (one extraction failed), and zero tested positive for wasp DNA.

2.4.3 Gut contamination

Eighteen cups containing beetles and slugs were tested for gut contamination. None of these cups contained ground beetles (n = 360) testing positive for slug DNA. The slug primers were used to amplify two types of extracted ethylene glycol samples prior to the addition of carabids (slug adjacent samples; slug container samples). Slug DNA was amplified in 17 of 18 slug adjacent samples, and 0 out of 18 slug container samples.

2.5 Discussion

Due to its sensitivity, molecular gut-content analysis has the capacity to detect trace amounts of prey DNA among copious quantities of predator DNA. However, this sensitivity makes PCR susceptible to contamination, requiring careful consideration of the inherent risks of misinterpreting trophic relationships. We tested for surface and gut-content contamination and revealed that zero specimens stored together in alcohol had surface contamination and zero pitfall-simulated containers had contamination in the beetles collected in them. Shokralla et al. (2010) documented that specimens stored in ethanol for only 24 h exuded amplifiable DNA. They tested only the preservative fluid and did not explore whether this DNA contaminated other specimens.

We amplified slug DNA from slug adjacent ethylene glycol samples after 24 h exposure to slugs, but this was not transferred to beetle gut-contents. Our research suggests that researchers may use wet pitfall traps without fear of contamination, but further studies are required to determine that this same lack of contamination occurs with other preservative fluids such as propylene glycol, often used for minimal vertebrate toxicity (LaKind et al. 1999). We would expect a lack of gut contamination to be found with propylene glycol, as it has similar DNA preservation to ethylene glycol (Aristophanous 2010), but simple pilot studies could be implemented to answer this question.

Fluid-containing pitfall traps are efficient for mass-collecting ground predators (e.g., carabid beetles, spiders, etc.) but have the disadvantage of inadvertently collecting non-target specimens in the same vicinity. For example, 20.6% of pitfall traps targeting ground beetles used in a strawberry study contained slugs (Eskelson et al. 2011). Should such inadvertent trapping cause significant false-positive gut contamination of specimens, safeguards such as calibration terms would be required when inferring food web connections. We amplified slug DNA from slug adjacent ethylene glycol samples from 17 of the 18 experimental units but could not amplify slug DNA from any of the 18 slug container ethylene glycol samples. Although the ethylene glycol was directly contaminated, contamination was not found in the guts of any ground beetles tested, suggesting that this is not a viable avenue for gut contamination in the time frame that we tested. Trap fluid preventing predator feeding activities in the trap potentially contributes to the mechanism of contamination avoidance (King et al. 2012). In addition, we used an organism that represented the worst-case scenario as slugs exude mucus and were able to

readily contaminate the preservative fluid without leading to gut-content contamination. This suggests that wet pitfall traps may be a safe way to collect predators for molecular gut-content analysis.

We stored two insects together in ethanol for up to 120 days and found no surface-level contamination. A previous study documented surface contamination from forceful collecting methods such as shaking plants and aspirating insects en masse into a container with false-positive rates found to be as high as 31% (Greenstone et al. 2011). Our experiment was conducted in the laboratory and specimens were handled carefully to ensure that any surface contamination would have occurred as a result of being stored together, not from sample collection. Our specimens were also dead when they were placed in the vials, insuring that if we had found contamination, it was only from storing them together and not from them damaging each other when dying in a preservative fluid.

This research provides no evidence of false positives generated by molecular gut-content analysis by storing specimens together in alcohol for extended periods of time or by using simulated pitfall traps. As the number of studies using these techniques continues to increase, methodological studies provide an important framework upon which sampling procedures should be developed. Care should always be exercised when collecting and storing samples for subsequent molecular analysis to avoid, or minimize, the likelihood for cross-contamination of external body parts or gut-contents, but can be optimized for ease of collection. Molecular gut-content analysis will continue to grow in use with the reduced cost of next-generation sequencing techniques allowing us to have a much broader picture of food webs and optimizing collection techniques will be crucial to our understanding of these relationships.

Table 2-1 COI primers used in this study.

Taxon	Primer pair	Primer sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)	Primer sensitivity (pg μl^{-1})
Campoletis sp.	Camp360-F	TTAATCATGAAGGTATATCAGTTGATTTAT	208	60	0.10
	Camp567-R	GCACCAGCTAAAACTGGTACTGC			
Drosophila melanogaster	Dros18-F	TTGGAGCTTGAGCTGGAATAG	220	61	6.25
	Dros237-R	GGGAATGCTATATCAGGAGCA			
Philomycidae	Phylo-32-F	GTGGAATAGTCGGTACAGGMYTATC	288	60	0.05
	Phylo-322-R	CAGCACCACCTTCTACTATTCTAGAAC			

Chapter 3: Stage specific aggregation mediates density dependent prey responses to non-consumptive predator effects

Chapter contents submitted as Athey, K.J**.,** Sitvarin, M.I, Harwood, J.D. 2017. Stage specific aggregation mediates density dependent prey responses to non-consumptive predator effects. *Environmental Entomology.*

3.1 Summary

Conservation biological control manipulates habitat characteristics to enhance natural enemy populations and ultimately reduce pest density. These practices can be most effective early in the growing season when pest populations are low. Early season predator impacts on these pests can include both direct consumption of herbivores and non-consumptive effects such as superfluous killing, both of which provide pest suppression. We combined a field cage experiment with molecular gut-content analysis to explore the effects of striped lynx spiders (*Oxyopes salticus* Hentz) on brown marmorated stink bugs (*Halyomorpha halys* (Stål)). To simulate field conditions in both early and late season, we manipulated the density and relative abundance of stink bug nymphs and adults in the presence and absence of lynx spiders. Interestingly, at high stink bug densities, more live stink bug nymphs were recovered from field cages with spiders, whereas adults had lower recovery rates. For nymphs, this result may be due to their response to aggregation pheromones, whereas highly mobile adults likely encountered spiders more frequently, promoting superfluous killing by spiders. Although dead stink bugs were recovered, we found no evidence of consumption through molecular gut-content analysis, strengthening evidence for lethal non-consumptive predator effects. Contrary to expectations that generalist predators would have the greatest impact on pests

early in the growing season, our results support stronger predator-prey interactions when pest densities are highest. Biological control might be most effective when utilizing multiple predator species as part of a complex of natural enemies, so spiders acting in concert with other generalist predators could be capable of suppressing pest populations.

3.2 Introduction

The agricultural landscape, often characterized by a low abundance and diversity of arthropods, can be inhospitable for generalist natural enemies and often does not supply the additional resources these animals require for survival, growth, and development (Landis et al. 2000). These resources include extra-floral nectaries (Baggen and Gurr 1998, Heil 2015), refugia (Halaj 2000, Knapp and Rezac 2015), and alternative prey and hosts (Gurr et al. 2004, Chapman et al. 2013). To ameliorate these challenges, conservation biological control involving habitat manipulation to enhance natural enemies and increase their effectiveness against pests can be utilized (Landis et al. 2000). This management approach can also serve to reduce mortality of natural enemies in agroecosystems through a reduction in pesticide sprays (Gurr et al. 2004) and these practices can be most effective early in the season, when alternative prey and refugia are scarce.

Early in the growing season, agricultural fields are characterized by rapid emergence, dispersal, and growth of pests and natural enemies. Interactions among organisms at this critical time can have long-lasting effects on the populations of these taxa later in the year (Fleming 1980, Landis and Van Der Werf 1997, Landis et al. 2000, Fox et al. 2005). For example, alternative prey allowed the predatory bug, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae), to establish in soybean fields early in the

season, thereby increasing their numbers sufficiently to exert some degree of control over soybean aphids, *Aphis glycines* Matsumura (Hemiptera: Aphididae), upon their initial colonization of the field (Yoo and O'Neil 2009). During this time, manipulation of generalist predator populations through conservation biological control is critical for maintaining pests at low levels and/or delaying the time at which pest populations reach economic thresholds (Welch and Harwood 2014, Athey et al. 2016). Generalist predators do not typically exert control on pest species when pest populations reach a maximum, and the lack of synchrony between pest populations and generalist predator diets is sometimes purported as detrimental to biological control. However, effective pest control most likely occurs when the ratio of predators to pests is greatest, facilitating a significant impact on pest population growth trajectories (Welch and Harwood 2014, Athey et al. 2016), likely due to significant predation on scarce pest prey (Harwood et al. 2007a, Harwood et al. 2009, Chapman et al. 2013).

Measuring the effect of generalist predators in open field conditions can be challenging due to the myriad of interactions between biotic and abiotic events (Welch and Harwood 2014). However, molecular gut-content analysis employing polymerase chain reaction (PCR) has become a popular tool for deducing the strength of trophic links in agroecosystems (e.g. Harwood et al. 2007b, King et al. 2011, Traugott et al. 2012, Hagler and Blackmer 2013, Schmidt et al. 2014). This has facilitated insights into feeding patterns when gut-contents cannot be morphologically identified. In addition, molecular gut-content analysis can expose the decoupling of prey availability and consumption, which cannot always be detected when relying on abundance data alone (Chapman et al. 2013, Gomez-Polo et al. 2014).

Although these approaches allow reliable post-mortem identification of prey items contained in the gut of the predator, they do not alone quantify biological control service. Combining molecular gut-content analysis with ecological experimentation (e.g., manipulation of predators and prey in field cages) can provide a more complete understanding of predator-prey interactions. Specifically, non-consumptive predator effects (NCEs) may contribute significantly to predator impacts on prey populations (Preisser et al. 2005), yet these NCEs would not be detectable using molecular gut-content analysis. Molecular methods are unlikely to detect superfluous (or wasteful) killing, a behavior whereby predators may abandon prey after attack (Maupin and Riechert 2001). Many groups of animals exhibit this behavior, including tardigrades, (Hohberg and Traunspurger 2009), mammals, (Short et al. 2002), insects (Johnson et al. 1975, Lang and Gsodl 2003, Lounibos et al. 2008) and spiders (Riechert and Maupin 1998, Maupin and Riechert 2001, Trubl et al. 2011).

Spiders are abundant in agroecosystems, constituting some of the most prevalent generalist predators in temperate areas (Wise 1993). They are often food limited in the field (Bilde and Toft 1994) but can survive under these conditions for a considerable time (Anderson 1974), making them important natural enemies in agroecosystems due to temporal variability in food availability. These characteristics could therefore allow spiders to exert a substantial impact on pest populations early in the season when pest numbers are typically low (Chiverton 1987, Sunderland et al. 1987, Harwood et al. 2004, Harwood et al. 2007a, Chapman et al. 2013) potentially maintaining pest outbreaks below economic thresholds. Furthermore, if a common prey type is distasteful, leading to aversion (e.g., Toft 1997, Sunderland 1999), spiders may kill more prey than they

consume, resulting in an increased rate of prey killed per spider (Sunderland 1999). In addition, superfluous killing is usually observed when prey are plentiful (Sunderland 1999). This form of induced prey mortality through superfluous killing would be underestimated by molecular gut-content analysis, thus reducing the assumed pest control services provided by spiders. Therefore, a combination of approaches is necessary to fully ascertain the impact of these predators on pest populations.

We utilized brown marmorated stink bug (*Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae)) as a "distasteful" pest species (Aldrich 1995). *Halyomorpha. halys* is invasive in the United States, being native to China, Korea, and Japan, was first reported in 1996 in Allentown, Pennsylvania, and subsequently identified in 2001 (Hoebeke and Carter 2003). It is recognized as a pest of many important crops and an urban nuisance given its proclivity to overwinter within houses (Hoebeke and Carter 2003). Since its entrance into the United States, *H. halys* has spread quickly and is currently found in 43 states (StopBMSB 2017). Striped lynx spiders (*Oxyopes salticus* Hentz (Araneae: Oxyopidae)) are dominant predators of many agricultural pests (Young and Lockley 1985, Young and Edwards 1990, Nyffeler et al. 1992). They are ambush predators and frequently are found on plants. There are several studies employing molecular gut-content analysis showing *O. salticus* readily consuming several species of stink bugs (Greenstone et al. 2014a) suggesting the potential for these spiders to exert some degree of control over *H. halys*, providing a pest control service in agroecosystems.

We combined experimental field cage manipulation with molecular gut-content analysis to assess the impact of a generalist predator on an agricultural pest. We predicted

that spiders, possessing the propensity for superfluous killing, interacting with stink bugs known to have characteristics that promote superfluous killing (distasteful and occurring at high densities) would contribute to biological control through non-consumptive interactions. In addition, we hypothesized that spiders would engage in superfluous killing when the stink bugs were at a higher density.

3.3 Materials and Methods

3.3.1 Field Cage Study

The experiment was conducted at the Spindletop Research Farm in Lexington, Kentucky, USA (38.1272° N, 84.5081° W). Field cages were 1.83 m x 1.83 m x 1.83 m, covered with nylon mesh screening (52 × 52 mesh count) to allow light, wind and rainfall penetration, but prevent arthropod entry or escape, with a side zipper for access and tent stakes to anchor the cages into the ground (Lumite Inc, Alto, Georgia, USA). Cage bottoms were buried ca. 20 cm into the soil to prevent arthropod entry/exit. To remove alternative prey that may be present in the field, a leaf blower (Poulan Pro 25cc Gas Blower/Vac, Poulan, Charlotte, North Carolina, USA) modified with an insect net attached to the intake was used to collect all arthropods present in the cages before the beginning of the experiment. Each cage was placed over three, 76 cm rows of full season, group 4.7 soybean plants (Asgrow AG4703) (Monsanto Company, St. Louis, Missouri, USA). Each cage consisted of approximately 110 mature soybean plants. BMSB and lynx spiders were collected from Spindletop Research Farm and maintained in the greenhouse prior to experimentation under controlled conditions (25 ± 1°C, 65 ± 5% RH, and photoperiod of 16:8 (L:D) h). *Halyomorpha halys* were maintained on a diet of green bell peppers and carrots and the lynx spiders were provided *Drosophila melanogaster* Meigen

(Diptera: Drosophilidae) but deprived of food for 48 h prior to the experiment to standardize feeding motivation.

Five treatments were used to evaluate the effect of *O. salticus* on *H. halys* (Table 3-1, n = 5/treatment). The two treatments with *O. salticus* and *H. halys* simulated natural densities of this pest. Henceforth, the 3-level food chain treatments with 17 and 37 *H. halys* will be referred to as low density and high density, respectively (Table 3-1). *Oxyopes salticus* numbers were representative of population levels in agricultural systems (Nyffler and Sunderland 2003), including soybean fields in the region of study (Athey & Harwood, unpublished data).

Halyomorpha halys (2^{nd} instar nymphs and a mixture of adult males and females) were added to the cages and given 48 h to acclimate, after which *O. salticus* were added. The experiment ran for five days, with *O. salticus* present for three, followed by destructive sampling, which consisted of removing all plant material from each cage, bringing it into the lab and recover all dead and living *H. halys* and *O. salticus*. All recovered *O. salticus* and *H. halys* were placed individually in 1.5 mL microcentrifuge tubes with 95% ethanol and stored at -20°C for subsequent molecular gut-content analysis.

3.3.2 Sequencing for Primer Design

To obtain sequences for primer design, DNA was extracted from specimens using DNeasy Blood and Tissue Kits© (Qiagen Inc., Valencia, California, USA) following standard animal tissue protocols. DNA was then amplified using general 16S primers; 16Sbr-H (5'- CCG GTC TGA ACT CAG ATC ACG T -3') and 16Sar-L (5'- CGC CTG

TTT AAC AAA AAC AT -3') (Palumi et al. 1991). Polymerase chain reactions (PCR) consisted of 1X Takara buffer (Takara Bio Inc., Shiga, Japan), 0.2 mM of each dNTP, 0.2 mM of each primer, 1.25 U Takara Ex Taq™ and template DNA (1-2 μL of total DNA). PCRs were carried out in Bio-Rad C1000 thermal cyclers (Bio-Rad Laboratories, Hercules, California, USA). The PCR cycling protocol was 94 °C for 1 min followed by 50 cycles of 94 °C for 45 s, 40 °C for 45 s, 72 °C for 45 s and a final extension of 72 °C for 5 min. PCRs included a positive control and a negative control consisting of all reagents without the addition of DNA. Following amplification, the bands were visualized on 2% SeaKem agarose (Lonza, Rockland, Maine, USA) pre-stained with GelRed nucleic acid gel stain (1X; Biotium, Hayward, California, USA). DNA sequencing was carried out at Advanced Genomics Technologies Center (University of Kentucky, Lexington, Kentucky, USA).

3.3.3 Primer Design

To design a primer to test for predation on *H. halys*, sequences (GenBank accession numbers KT189171-KT189179) were edited using Geneious© (Biomatters Ltd, Auckland, New Zealand) and aligned using MUSCLE (Edgar 2004). We designed primers by visually inspecting the sequences using BioEdit 7.0.0 (Isis Pharmaceuticals Inc., Carlsbad, California, USA) and using Primer3 (Rozen and Skaletsky 2000) to determine whether the primer properties were adequate. The *H. halys* primers were: BMSB-34F (5’- AAC ATG TCC TAA TGA TTA ATT AG -3’) and BMSB-149R (5’- TAT AAA GAA AGA TAT TCC TTC ATC CG -3’) producing a 156 bp amplicon. All reaction conditions were identical to the 16S primer protocol, except the PCR cycling conditions were 94 °C for 1 min followed by 40 cycles of 94 °C for 45 s, 60.5 °C for 45

s, 72 °C for 30 s. Primers were screened against 183 nontarget taxa (Table 3-2). All *O. salticus* recovered from the cages at the end of the experiment were screened for *H. halys* predation using the *H. halys* specific primers.

Primer sensitivity was determined by testing dilutions of target DNA for amplification. DNA concentration was determined using a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany) adjusted to 5000 pg/µL and two-fold serially diluted. The serially diluted target DNA was used as a template at concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, 0.05, 0.025 pg/µL of target DNA.

3.3.4 Feeding Trials

Feeding trials were conducted to determine the detectability half-life of DNA within the gut of the spiders (Greenstone et al. 2014b). *Halyomorpha halys* nymphs were maintained in a greenhouse in similar conditions to the field cage specimens. *Oxyopes salticus* were collected from Spindletop Research Farm and maintained under controlled conditions at 25°C, 16:8 LD. *Oxyopes salticus* were deprived of food for a minimum of 48 hours before encountering *H. halys*. *Oxyopes salticus* were maintained in 162 mL plastic cups with 5 mm of plaster in the bottom for moisture retention. Once *O. salticus* attacked *H. halys*, it was allowed to feed for 2 hours. Feeding was observed so that we could confirm duration of feeding. After the 2 hour feeding time, what was left of the *H. halys* was removed and the *O. salticus* was maintained until its scheduled freezing time. The freezing time intervals were 0, 1, 2, 4, 8, 10, 12, 16, 24, 48, 72, and 96 hours post feeding (n = 8 per time period). All *O. salticus* were placed into chilled 95% ethanol and frozen at -20°C for subsequent molecular analysis. If the *O. salticus* did not attack the

nymph within 4 hours, the *H. halys* was removed. *Oxyopes salticus* were presented with *H. halys* each day, following the procedures above, until they attacked and consumed one. This results in *O. salticus* that differ in their total food deprivation period.

3.3.5 Data Analysis

At the end of the experiment, we measured the proportion of *H. halys* nymphs and adults recovered (alive or dead) as well as the proportion of live *O. salticus* recovered. Analysis of variance was conducted followed by Dunnett's test to compare *H. halys* recovery in the low and high density treatments against the treatment lacking *O. salticus*. We used the same test to compare *O. salticus* recovery in the low and high density treatments against the treatment containing only *O. salticus*. The analyses were conducted in SAS 9.3 (SAS Institute, Cary, North Carolina, USA). The detectability of *H. halys* within *O. salticus* guts over time in the feeding trials was calculated using a Probit model in SAS 9.3.

3.4 Results

3.4.1 Field Cage Study

Overall, the percentage of *H. halys* recovered alive at the end of the experiment was low, varying between 37% and 58% of individuals remaining. We have no reason to believe that the numbers of individuals escaping during this time would have differed by treatment, as all plants were handled similarly and processed in a random order. There were also no *H. halys* recovered from the soybean only treatments suggesting that there was no immigration of *H. halys* into the field cages.

There were three possible outcomes for the *H. halys*: recovered alive, recovered dead or not recovered. There was no overall effect of treatment on recovery of *H. halys* nymphs ($F_{2, 12} = 3.4344$, $p = 0.07$). However, a higher proportion of living *H. halys* nymphs were recovered in the high density treatment compared with the *H. halys* only treatment ($d = 2.502$, $p = 0.041$). There was no effect of treatment on the proportion of dead nymphs recovered ($F_{2, 12} = 1.3333$, $p = 0.3$) the proportion of dead nymphs was similar between the high density and *H. halys* only treatments ($d = 2.502$, $p = 0.188$) (Fig. 3-1). Additionally, the total proportion of recovered nymphs, alive or dead, was similar between the low density treatment and the *H. halys* only treatment ($d = 2.502$, $p = 0.303$; $d = 2.502$ $p = 0.267$, respectively) (Fig. 3-1).

There was no overall effect of treatment on the proportion of living adult *H. halys* recovered ($F_{2,12} = 0.05$, $p = 0.9514$). The proportion of living adult *H. halys* was comparable between the *H. halys* only treatment and both high and low density treatments ($d = 2.502$, $p = 0.850$; $d = 2.502$, $p = 0.921$, respectively). There was also no overall effect of treatment on proportion of dead adults recovered ($F_{2,12} = 3.2632$, $p = 0.0739$) a marginally higher proportion of dead adults were recovered from the high density cages compared to the *H. halys* only treatment ($d = 2.502$, $p = 0.06$) (Fig. 3-2). However, there was no difference between the low density and *H. halys* only treatments ($d = 2.502$, $p = 0.792$) (Fig. 3-2).

In the absence of prey, the proportion of *O. salticus* recovered did not differ from those in the high or low density treatments ($d = 2.502$, $p = 0.991$; $d = 2.502$, $p = 0.816$, respectively) (Fig. 3-3).

3.4.2 Feeding Trials

The DNA detectability half-life of the amplicon for our *H. halys* primer within the guts of *O. salticus* was 8.2 hours (Fig. 3-4). At 72 h post feeding, the DNA was no longer detectable in the guts of *O. salticus*. The degradation rate for *H. halys* DNA was significantly different from zero ($\chi^2 = 8.58$, $p = 0.0034$). Over two-thirds (69.6%) of *O. salticus* did not attack a *H. halys* until they had not eaten for 72 h. Several *O. salticus* did not consume *H. halys* until they had been deprived of food for 189 h (Table 3-3).

3.4.3 Primer Design and Gut-Content Analysis

During characterization, the *H. halys* -specific primers had 100% amplification success for *H. halys* but elicited no amplification when screened against 183 non-target organisms from 78 families (Table 3-2). The primer sensitivity is 12.5 pg/µL. In total, 67 *O. salticus* recovered from the cages were tested for *H. halys* DNA, with none testing positive.

3.5 Discussion

Oxyopes salticus effects on *H. halys* varied with prey density, with high densities leading to an increased recovery of nymphs but reduced adult survival. This result may be explained by the life history and behavior of stink bugs. Aggregation is common in all life stages (Inkley 2012, Reay-Jones 2014) and first instar nymphs remain together on the egg mass until they molt (McPherson and McPherson 2000). Lockwood and Story (1986) demonstrated that aggregated nymphs of southern green stink bug (*Nezara viridula* (L.)) (Hemiptera: Pentatomidae) suffered lower mortality from generalist predators than non-aggregated nymphs, suggesting that aggregation may play an important role in their defense. Our demonstration of a higher proportion of nymphs recovered at higher density

may be due to the increased effectiveness of aggregation as more individuals aggregated, providing support for the dilution effect: the probability of an individual being attacked by a predator decreases as the size of an aggregation increases (Lehtonen and Jaatinen 2016). The proportion of nymphs recovered was highest in the high density treatment, suggesting that aggregation reduced the number of nymphs killed by spiders or otherwise not recovered. In the low density treatment, spider-induced mortality did not differ from that in the *H. halys* control (lacking *O. salticus*), indicating that the effects of spiders were compensatory (*sensu* Beckerman et al. 1997) to background mortality levels.

Although both stink bug nymphs and adults respond to aggregation pheromones, the life stages differ markedly in mobility and dispersal potential. Second instar nymphs (used in our experiment) walk distances comparable to adults (Lee et al. 2014), but adults readily fly long distances (Wiman et al. 2015). Highly mobile adults may have had increased encounter rates with spiders, which would increase the likelihood of being attacked by a visually-orienting predator such as *Oxyopes spp.* (Hu et al. 2014). We found no molecular evidence for consumption of stink bugs by spiders in our field cages; however, more dead adults were recovered from the high density treatment, suggesting that spiders facilitated stink bug mortality when at stink bugs were at high density without actually consuming them.

Our feeding trials showed the DNA half-life for our amplicon was 8.2 h, considerably shorter than the length of the experiment. It is possible that we were still observing non-consumptive effects and not missing the detection of consumption. In our feeding trials, 70% of *O. salticus* that fed on *H. halys* did not attack a stink bug until they had not eaten for more than the 72 h that our field cage experiment ran. This suggests

that the higher proportion of dead adults found in the high density treatments was evidence of a non-consumptive effect. We found no spiders testing positive for *H. halys* DNA and given the time it took for an attack to occur in our feeding trials, if consumption were taking place it was likely to have resulted in at least a few *O. salticus* test positive for *H. halys*. Our laboratory feeding trial suggests it is unlikely consumption occurred but was undetected, it is likely that NCEs, such as superfluous killing, are responsible for the higher proportion of dead adult stink bugs in the high *H. halys* density treatments.

Adult stink bugs are 2-3 times larger than lynx spiders (Dondale and Redner 1990, Hoebeke and Carter 2003) but these predators readily attack prey items much larger than themselves (Walker and Rypstra 2002, Nyffeler and Pusey 2014). Spiders may not have consumed the stink bugs because they are chemically defended (Millar 2005), but if spiders attacked highly mobile adults, venom injection and associated injury may have facilitated the increased mortality of dead adults found in the high density treatment. The same patterns were not present at low stink bug density, which is consistent with superfluous killing typically occurring when prey are highly abundant (Johnson et al. 1975, Sunderland 1999). Furthermore, spiders are more likely to engage in superfluous killing when the most abundant prey type is chemically defended (Sunderland 1999). The combination of increased encounter rate between spiders and mobile adults, high prey density, and chemical defense all may have led *O. salticus* to frequently attack adult *H. halys* without actually consuming them. Such behavior is consistent with the lack of molecular evidence for consumption during these experiments.

Our results suggest that *O. salticus* are unlikely to exert substantial early season control over *H. halys*. Additionally, no molecular evidence for consumption of *H. halys* by *O. salticus* was found in the field cages, and we only found evidence for superfluous killing in the high density treatment designed to replicate late season prey levels. Field studies have demonstrated that generalist predators can suppress pests if they are present early in the season prior to large increases in prey populations (e.g., (Landis and Van Der Werf 1997); reviewed by (Welch and Harwood 2014, Athey et al. 2016)). *Oxyopes salticus* may have been less effective at low prey densities because we standardized the number and age of soybean plants present in the field cages, meaning that spiders had an equivalent quantity of plant material to search regardless of prey density. In agricultural fields, early season plants would be smaller thus providing less habitat structure as refuge from predation. *Halyomorpha halys* recovery likely would have been lower if the low density treatment had smaller or fewer plants, as habitat structure is known to decrease predator-prey encounter rates (Birkhofer et al. 2008, Grabowski et al. 2008, Vucic-Pestic et al. 2010).

Overall, our results suggest that consumption of *H. halys* by *O. salticus* in the field is likely to be negligible (or absent altogether), and that this generalist predator is unlikely to exert substantial early season control. However, our field cages excluded other generalist predators and parasitoids, which would act in concert with *O. salticus* and potentially contribute to an effective conservation biological control regime. Enhanced predator diversity has been shown to contribute to increased pest suppression (Snyder et al. 2006, Straub and Snyder 2008), and spiders are likely to provide more effective pest control when natural assemblages are used instead of single species

(Riechert and Bishop 1990, Riechert and Lawrence 1997). Thus, the inclusion of multiple predator species may provide early season control of *H. halys*, preventing late season population outbreaks. Future studies examining suites of generalist predators may reveal productive directions for conservation biological control programs targeting *H. halys*.

Table 3-1. Treatments used in the field cage experiment, detailing the numbers of *Oxyopes salticus* (spider) and *Halyomorpha halys* (brown marmorated stink bug) present.

Treatment	**# of *O. salticus***	**# of *H. halys***
Soybean control	0	0
Predator control	7	0
2-level food chain, low density	0	7 adult + 10 nymphs
3-level food chain, low density	7	7 adult + 10 nymphs
3-level food chain, high density	7	7 adult + 30 nymphs

Table 3-2. List of non-target taxa screened for cross reactivity with *Halyomorpha halys* primer.

Order	Family	Species	No. Tested
Araneae	Araneidae	*Magora* sp.	1
	Araneidae	*Neoscona crucifera* (Lucas)	1
	Araneidae		3
	Linyphiidae	*Erigone autumnalis* Emerton	1
	Linyphiidae	*Glenognatha foxi* (McCook)	1
	Linyphiidae	*Tennesseellum formica* (Emerton)	1
	Lycosidae		2
	Miturgidae	*Cheiracanthium* sp.	1
	Oxyopidae	*Oxyopes* sp.	2
	Salticidae	*Hentzia mitrata* (Hentz)	1
	Salticidae	*Pelegrina proterva* (Walckenaer)	2
	Salticidae		1
	Tetragnathidae		2
	Thomisidae	*Misumena* sp.	1
	Thomisidae		1
	Unidentified		11
Coleoptera	Aderidae		1
	Anthicidae	*Notoxus* sp.	2
	Anthicidae	*Acanthinus argentinus* (Pic)	1
	Carabidae	*Lebia viridis* Say	1
	Carabidae		2
	Chrysomelidae	*Diabrotica undecimpunctata* (L.)	1
	Chrysomelidae		1
	Coccinellidae	*Coccinella septempunctata* (L.)	1
	Coccinellidae	*Coleomegilla maculata* De Geer	1
	Coccinellidae	*Hippodamia convergens* Guérin-Méneville	1
	Coccinellidae	*Scymnus* sp.	1
	Coccinellidae	*Hippodamia* sp.	2
	Coccinellidae	*Coccinella* sp.	1
	Curculionidae	*Hypera brunneipennis* (Boh)	1
	Curculionidae	*Hypothenemus hampei* Ferrari	1
	Elateridae		1
	Lathridiidae		1
	Latridiidae		1
	Meloidae	*Epicauta* sp.	1
	Melyridae	*Collops* sp.	1

Table 3-2 (continued)

Order	Family	Species	No. Tested
	Nitidulidae		1
	Phalacridae		1
	Staphylinidae		2
Diptera	Agromyzidae		1
	Anthomiidae		1
	Anthomyzidae		1
	Brachycera		6
	Chironomidae		1
	Chloropidae		1
	Dolicopodidae		2
	Drosophilidae	*Scaptomyza* sp.	1
	Drosophilidae		2
	Empididae		1
	Ephydridae		1
	Heliomyzidae		1
	Lonchopteridae		1
	Muscidae		1
	Mycetophilidae		1
	Nematocera		3
	Phoridae		1
	Syrphidae		3
	Tipulidae		1
Hemiptera	Aleyrodidae	*Bemisia tabaci* (Gennadius)	1
	Alydidae		3
	Anthocoridae	*Orius albidipennis* (Reuter)	1
	Anthocoridae	*Orius* sp.	1
	Aphididae	*Capitophorus eleagni* (Del Guercio)	1
	Aphididae	*Uroleucon gravicorne* (Patch)	1
	Aphididae		1
	Cicadellidae		4
	Coccidae	*Coccus hesperidum* (L.)	1
	Coccidae	*Neolecanium cornuparvum* (Thro)	1
	Cydnidae	*Sehirus cinctus* (Palisot)	3
	Cydnidae		1
	Geocoridae	*Geocoris* sp.	4
	Geocoridae		1
	Lygaeidae	*Nysius* sp.	1
	Miridae	*Lygus lineolaris* (Palisot de Beauvois)	1
	Nabidae	*Nabis capsiformis* Germar	4

Table 3-2 (continued)

Order	Family	Species	No. Tested
	Nabidae		2
	Pentatomidae	*Euschistus servus* (Say)	3
	Pentatomidae	*Nezara viridula* (L.)	4
	Pentatomidae		2
	Pseudococcidae	*Pseudococcus maritimus* (Ehrhorn)	1
	Psyllidae	*Cacopsylla pyricola* (Förster)	1
	Psyllidae		1
	Reduviidae	*Zelus* sp.	1
	Reduviidae		2
	Rhyparochromidae		1
	Thyreocoridae		1
	Unidentified		3
Hymenoptera	Argidae		1
	Bethylidae	*Prorops nasuta* Waterston	1
	Bethylidae		1
	Braconidae	*Aridelus* sp.	1
	Braconidae	*Meteorus* sp.	1
	Braconidae	*Bracon* sp.	1
	Braconidae		5
	Ceraphronidae	*Aphanogmus* sp.	1
	Chalcididae		1
	Crabronidae	*Mimesa* sp.	1
	Crabronidae		1
	Eulophidae	*Phymastichus coffea* (LaSalle)	1
	Figitidae		2
	Formicidae	*Tapinoma* sp.	1
	Formicidae		1
	Ichneumonidae		3
	Platygastridae	*Trimorus* sp.	1
	Platygastridae		1
	Pompilidae		1
	Pteromalidae		1
Lepidoptera	Unidentified		1
Mantodea	Mantidae		1
Neuroptera	Chrysopidae		3
	Hemerobiidae		2
Orthoptera	Tettigoniidae		1
	Tettigoniidae		1
Psocoptera	Unidentified		1
Thysanoptera	Thripidae	*Frankliniella occidentalis* (Pergande)	1

Table 3-2 (continued)

Order	Family	Species	No. Tested
	Thripidae	*Thrips tabaci* L.	1
Stylommatophora	Polygyridae	*Mesodon zaletus* (Binney)	1
	Discidae	*Anguispira alternata* (Say)	1

Table 3-3. Food deprivation periods (h) of *Oxyopes salticus* (spider) and the number that ate *Halyomorpha halys* (brown marmorated stink bug) in the feeding trials.

Food deprivation period (hrs)	Number included in feeding trials
48	18
72	3
92	2
114	9
161	10
189	27

Figure 3-1. Mean proportion (±SE) of live (black bars) and dead (white bars) *Halyomorpha halys* (brown marmorated stink bug (BMSB)) nymphs recovered from field cages when present alone or in the presence of low or high densities of *Oxyopes salticus*. * Indicates significant difference when compared to the control (BMSB only) using Dunnett's test (d = 2.502, p = 0.041). Detailed treatment descriptions are given in Table 3-1.

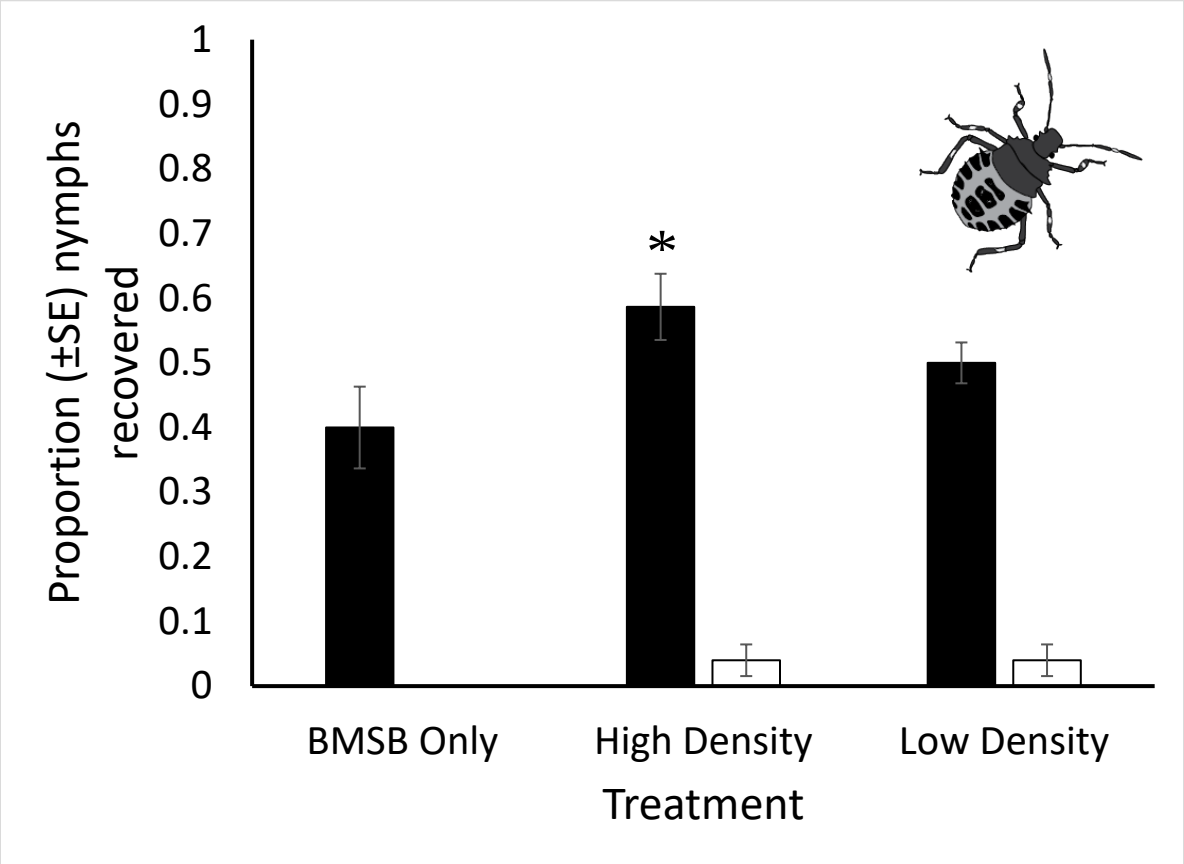


Figure 3-2. Mean proportion (±SE) of live (black bars) and dead (white bars) *Halyomorpha halys* (brown marmorated stink bug (BMSB)) adults recovered from field cages when present alone or in the presence of low or high densities of *Oxyopes salticus*. * Indicates marginal significant difference when compared to the control (BMSB only) using Dunnett's test (d = 2.502, p = 0.06). Detailed treatment descriptions are given in Table 3-1.

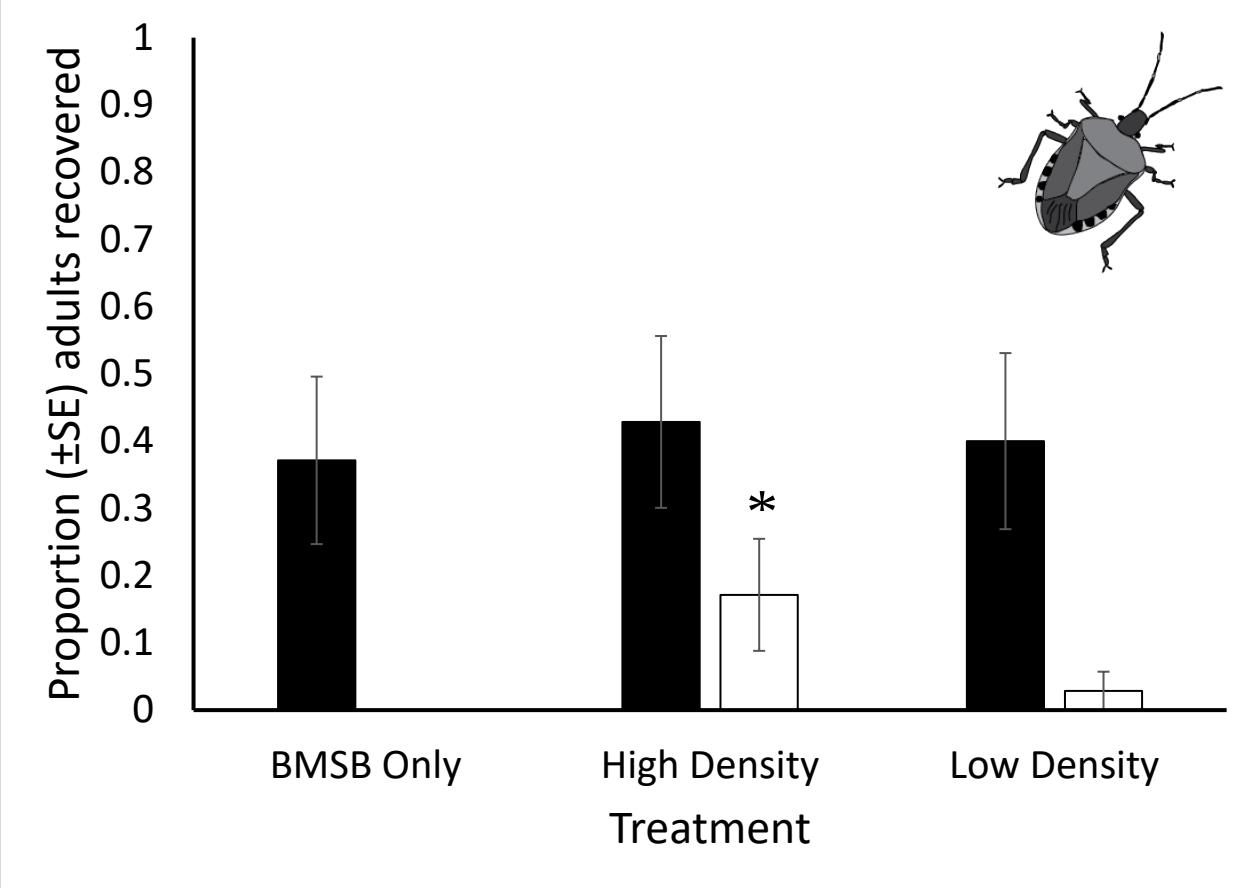


Figure 3-3. Mean proportion (±SE) of live *Oxyopes salticus* (lynx spiders) recovered from field cages when present alone or in the presence of low or high densities of *Halyomorpha halys* (brown marmorated stink bugs (BMSB)). Detailed treatment descriptions are given in Table 3-1. No significant differences between treatments.

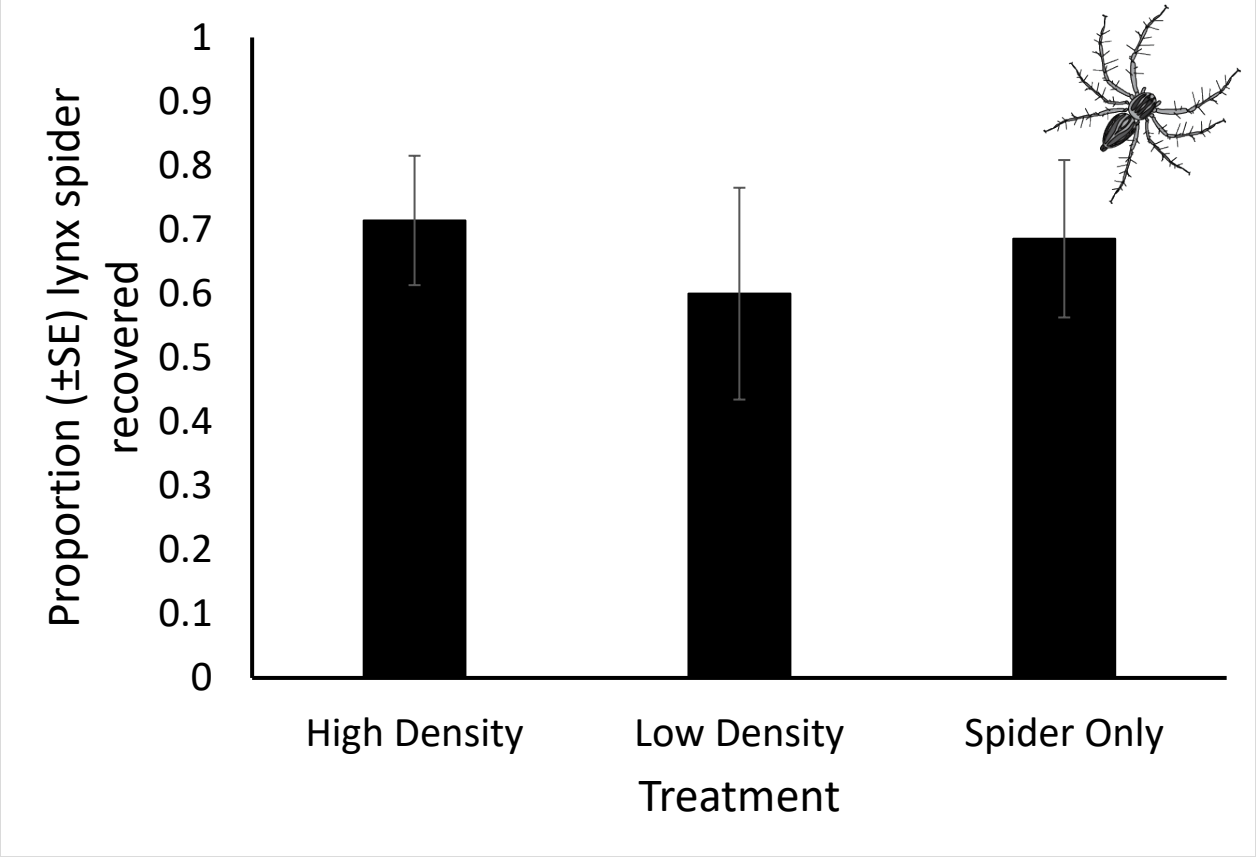
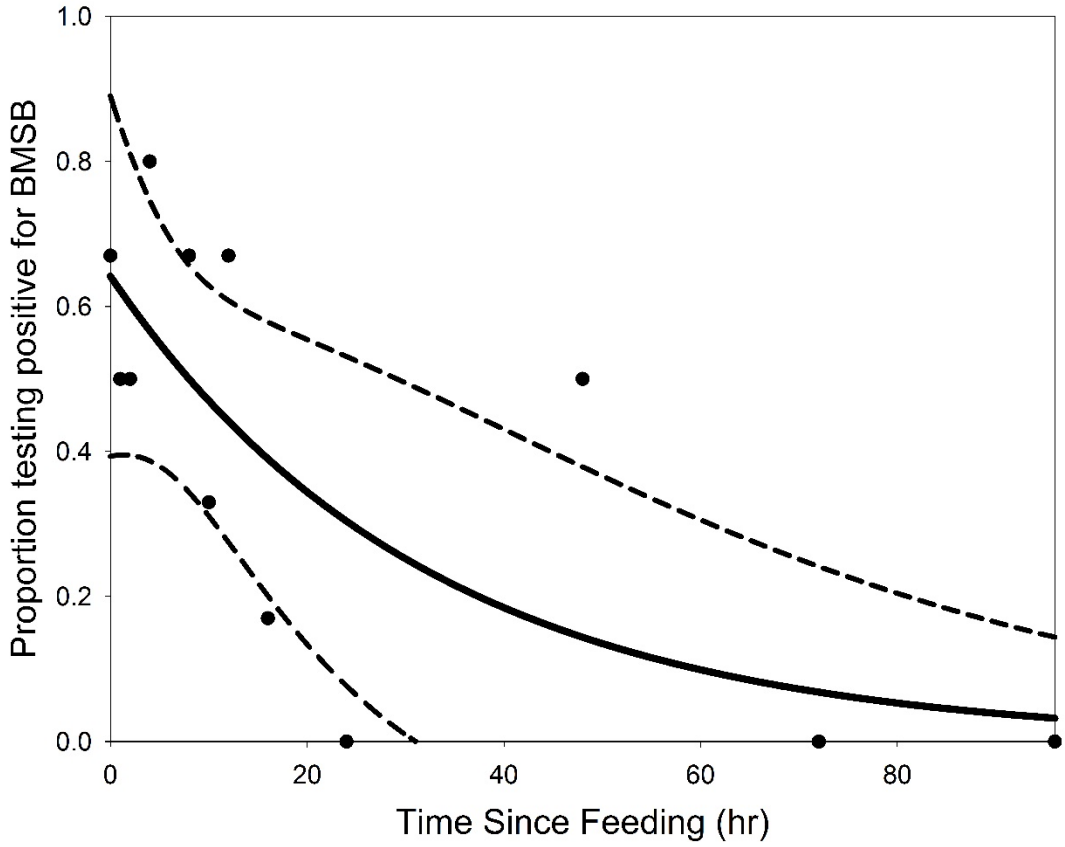


Figure 3-4. Results of the lynx spider feeding trial. Dots represent the proportion of samples at each time point (0-96 h) testing positive for *Halyomorpha halys* (brown marmorated stink bug (BMSB)) DNA. Dashed lines represent the upper and lower 95% confidence intervals.



Chapter 4: Predation on stink bugs (Hemiptera: Pentatomidae) in cotton and soybean agroecosystems

4.1 Summary

Stink bugs (Hemiptera: Pentatomidae) are significant pests of cotton and soybeans in the southeastern United States with annual control costs exceeding $14 million. Three of the most prominent pest species are the southern green, *Nezara viridula*, brown, *Euschistus servus* and green, *Chinavia hilaris*, stink bugs. To determine trophic linkages between generalist predators and these pests, species-specific 16S molecular markers were designed and used to detect the presence of prey DNA in predator gut-contents. Over 2700 predators were collected during two growing seasons in cotton and soybean in southern Georgia in 2011 and 2012 and screened for stink bug DNA. Trophic linkages were analyzed relative to prey availability, crop type and field location. *N. viridula* populations were significantly higher than *E. servus* and *C. hilaris* populations in both years. Predation was negligible on *E. servus* (0.23%) and *C. hilaris* (0.09%). Overall predation on *N. viridula* was 3.3% and *Geocoris sp.* (Hemiptera: Geocoridae), *Orius sp.* (Hemiptera: Anthocoridae) and *Notoxus monodon* (Coleoptera: Anthicidae) were the primary predators. This contrasts with previous studies that have found a much more diverse suite of predators consuming stink bugs with much higher gut-content positives. The discrepancy between studies highlights the need for replication studies, especially if the goal is to implement conservation biological control in integrated pest management.

4.2 Introduction

Phytophagous stink bugs (Hemiptera: Pentatomidae) are pests in cotton (Greene et al. 2001, Williams 2013) and soybean crops (Turnipseed and Kogan 1976, Panizzi and Slansky 1985). Historically, southern green stink bug, *Nezara viridula* (L.) and green stink bug, *Chinavia. hilaris* (Say) were the two most important stink bug pests in soybean in the southern United States and South America (Panizzi and Slansky 1985). However, these two species are joined by the brown stink bug (*Euschistus servus* (Say)) as a stink bug complex in the southern United States (McPherson and McPherson 2000). None of these stink bugs are directly affected by Bt (*Bacillus thuringiensis* Berliner) (Bacillales: Bacillaceae) toxins in transgenic cotton, currently in widespread use. Bt cotton use has led to decreased broad spectrum insecticide use in the southern United States (Ruberson et al., 2012). Further, the cotton boll weevil eradication program also reduced insecticide use. Historically, stink bugs were collaterally controlled by insecticidal sprays targeting other pests, and without these sprays, stink bugs have emerged as significant pests in crop systems (Turnipseed et al. 1995, Greene et al. 1999). Additionally, release from competition with Bt-targeted insects, like *Helicoverpa zea* (Boddie), may contribute to stink bug outbreaks in cotton (Zeilinger et al. 2016).

Stink bug species have been traditionally lumped together as a pest complex potentially making it difficult to assess their individual economic impact (Bundy and McPherson 2000, McPherson and McPherson 2000, Vyavhare et al. 2014). Different species of stink bugs can have differential impacts on cotton, with different levels of damage to bolls (Zeilinger et al. 2015) and soybean crops, in terms of damaged seeds (Corrêa-Ferreira and de Azevedo 2002), highlighting a need to study stink bugs as

individual species and not just as a pest complex. Their wide host ranges and varied feeding habits complicate the lumping together of the species with respect to their economic impacts. As noted, these stink bug species are pests of soybean and cotton, but are also pests in grain, fruit, nut and vegetable production where they cost millions of dollars in control and yield losses (McPherson and McPherson 2000). *Nezara viridula* is highly polyphagous, attacking over 30 species of plants (Todd 1989, Panizzi 2000, Panizzi et al. 2000). Jones and Sullivan (1982) showed that *C. hilaris* could utilize about 16 different host plants for development and reproduction. Several other species of stink bugs (e.g. *E. servus*, *E. tristigmus* (Say), *Thyanta accerra* McAtee) were found to exploit a number of hosts in addition to economically important crops, such as soybean and cotton (Jones and Sullivan 1982).

These stink bug species also vary in their susceptibility to insecticides (Willrich et al. 2003, Vyavhare et al. 2014). For example, *E. servus* was found to be less susceptible to some pyrethroids and organophosphates than was *C. hilaris* and *N. viridula* (Snodgrass et al. 2005). The variability in species-specific impact on crops, susceptibility to various insecticides, and general species ecology underscore the need for an integrated approach to managing stink bug pests.

Integrated pest management (IPM) programs benefit greatly from incorporating biological control (Naranjo 2011). Generalist predators contribute vital ecosystem services through pest control (Power 2010) and pest control utilizing natural enemies in the United States has been estimated to save $4.5 billion annually (Losey and Vaughan 2006). Many studies have identified predators of stink bugs (Yeargan 1979, Ragsdale et al. 1981, Stam et al. 1987, Van Den Berg et al. 1995, Ehler 2002, Tillman 2008, Tillman

2011, Olson and Ruberson 2012), but only two recent studies utilized PCR for identifying stink bug predators (Greenstone et al. 2014a, Tillman et al. 2015).

In a study using sentinel *N. viridula* egg masses in weeds, tomato and beans, predation varied from 0-68.2% of the total eggs. (Ehler 2002). Yeargan (1979) measured egg predation in soybean and alfalfa with up to 31 % and 50% of eggs consumed by sucking and chewing predators, respectively. Olson and Ruberson (2012) found that predation of *N. viridula* sentinel eggs masses, mainly by fire ants, *Solenopsis invicta* Buren (Hymenoptera: Formicidae) and longhorned grasshoppers (Orthoptera: Tettigoniidae), was crop specific where egg mortality was 74-86% in peanut, 39-65% in soybean and 4-34% in cotton. Additionally, Tillman (2011) identified predators observed on egg masses (sucking: *Podisus spp*. (Hemiptera: Pentatomidae), *Orius spp.* (Hemiptera: Anthocoridae), *Geocoris spp.*(Hemiptera: Geocoridae); chewing: ants and ladybeetles (Coleoptera: Coccinellidae)), but only quantified predation based on feeding mode.

Other researchers combined observations and radioactive labelling to assess predation on *N. viridula* in soybeans, with the dominant egg predators being *S. invicta* and grasshoppers (Stam et al. 1987). They also observed *Nabis spp.* (Hemiptera: Nabidae), phytophagous stink bugs, *Sinea sp*. (Hemiptera: Reduviidae) green lacewing larvae (Neuroptera: Chrysopidae) and an anthicid beetle (Coleoptera: Anthicidae) (Stam et al. 1987). In a study employing ELISA for detection of predation on *N. viridula* eggs and nymphs, predators from several species were tested and ladybeetles, *Geocoris punctipes*, *Orius insidiosus*, *Podisus maculiventris*, *S. invicta*, *Nabis roseipennis*, and *Lebia analis* (Coleoptera: Carabidae) were positive for egg predation (Ragsdale et al. 1981). Ladybeetles, *Oxyopes salticus*, (Araneae: Oxyopidae) *Phidippus audax* (Araneae:

Salticidae), *Neoscona arabesca* (Araneae: Araneidae), *G. punctipes*, and *N. roseipennis* were positive for stink bug nymph predation (Ragsdale et al. 1981).

Molecular gut-content analysis is a popular tool for determining trophic linkages (Juen and Traugott 2007, King et al. 2008, Furlong 2015, Hagler et al. 2015) and screening many diverse predators in a short amount of time for a given prey item. This technique is useful in agroecosystems (Hagler and Blackmer 2013, Schmidt et al. 2014) and has been used in stink bug food webs (Greenstone et al. 2014a, Tillman et al. 2015). The majority of terrestrial arthropod predators feed cryptically by liquid ingestion following extra-oral digestion (Cohen 1995), determining trophic linkages without molecular methodologies would require visual observations and not allow large number of predators to be screened quickly. There are however, limitations to molecular gut-content analysis, such as the inability to separate primary predation from secondary predation or scavenging (von Berg et al. 2012). In addition, molecular gut-content analysis using PCR is a strictly qualitative measure of predation (Greenstone et al. 2010) but if used in conjunction with prey abundance data may allow inference about the potential impact on the overall pest population (Furlong 2015).

For this project, we employed molecular gut-content analysis to determine which predators from a diverse suite of insects and spiders consumed three species of stink bugs in cotton and soybean crops in Georgia over two years in three locations. We tested for differences in predation as a function of crop types, farm locations and prey availability. The main objective of this study was to determine which predators most frequently preyed upon stink bugs at different times of the season in cotton and soybeans.

4.3 Materials and Methods

4.3.1 Field Conditions

Field sampling took place on soybean-cotton farms from July through October 2011 and 2012 in three locations in southwestern Georgia, USA. The locations were Belflower Farm, Tifton, GA (Tift Co.) (N31° 30.434 W083° 33.430) (planted on 2 June 2011, 17 June 2012), the Attapulgus Research and Education Center, Attapulgus, GA (Decatur Co) (N30°76.254 W84° 48.488) (planted on 31 May 2011, 17 June 2012) and the Southwest Research and Education Center, Plains, GA (Sumter Co) (N32° 03.589 W84° 36.691) (planted on 6 June 2011, 17 June 2012). In 2011, three crops at each location were sampled: Bt-cotton (DP1034B2RF), soybeans MG5 (maturity group 5) (Agsouth Genetics 568RR) and soybeans MG6.9 (maturity group 6.9) (Asgrow AG6931RR) (Monsanto Co, St. Louis, MO, USA). The different soybean maturity groups were used because they attract different complexes of predators (McPherson 1996). In 2012, four crops were sampled at each location: Bt-cotton, non-Bt cotton, MG5 soybeans and MG7 soybeans. Aldicarb (Bayer CropScience Leverkusen, Germany), was applied in furrow at planting in cotton (3.93 kg/ha (3.5 lbs/acre)) for thrips control (Grey et al. 2006). No other insecticides were applied. Because there were different crop types in the two years, each year was analyzed separately.

4.3.2 Arthropod Sampling

Samples were collected biweekly (2011: 29 July- 7 October, 2012: 12 July – 11 October) using a 31 cm diameter sweep net with 100 sweeps per sample, with two samples per field which were pooled for subsequent analyses. Within each field, samples were taken along two different rows separated from one another by six rows. Sweeping

was initiated five meters into the crop and along rows at least five rows from the plot edge to reduce edge effects. Different rows were sampled on each sample date to prevent prolonged disruption of sampling rows. All arthropods were counted with predators and stink bugs immediately separated and placed in sterile 1.5 mL microcentrifuge tubes filled with 95% ethanol. Specimens were identified to the lowest taxonomic level possible and then frozen at -20°C until subsequent DNA analysis.

For primer design, specimens of *N. viridula*, and *E. servus* were collected from lab colonies and field locations in Tifton, GA and non-target species were collected in field locations in Tifton, GA. Primers to amplify *C. hilaris* were designed in conjunction with a previous study (Penn et al. 2017). Each specimen was preserved as above.

4.3.3 Molecular Gut-content Analysis

Total DNA was extracted from all specimens using DNeasy Blood and Tissue Kits© (Qiagen Inc., Valencia, CA, USA) following standard animal tissue protocols. For primer design, stink bug legs were removed and DNA was extracted. For molecular gut-content analysis, all predators were crushed and whole body extracted (Table 4-1).

For primer design, we amplified 16S sequences using general primers; 16Sbr-H and 16Sar-L (Palumi et al. 1991). Polymerase chain reactions (PCR) (25 µL) consisted of 1X Takara buffer (Takara Bio Inc., Shiga, Japan), 0.2 mM of each dNTP, 0.2 mM of each primer, 1.25 U Takara Ex Taq™ and template DNA (1-2 µL of total DNA). PCRs were carried out in Bio-Rad PTC-200 and C1000 thermal cyclers (Bio-Rad Laboratories, Hercules, CA, USA). The PCR protocol was 94 °C for 1 min followed by 50 cycles of 94 °C for 45 s, 63 °C for 45 s, 72 °C for 45 s and a final extension of 72 °C for 5 min. PCRs

included a positive and negative control. Following amplification, the bands were visualized on 2% SeaKem agarose (Lonza, Rockland, Maine, USA) pre-stained with GelRed nucleic acid gel stain (1X; Biotium, Hayward, California, USA). The PCR product was purified and sequenced at AGTC (University of Kentucky, Lexington, KY, USA).

Sequences were edited using Geneious© (Biomatters Ltd, Auckland, New Zealand) and aligned using MUSCLE (Edgar 2004). We designed primers by visually inspecting the sequences using BioEdit 7.0.0 (Isis Pharmaceuticals Inc., Carlsbad, CA, USA) and then using Primer3 (Rozen and Skaletsky 2000) to determine whether the primer properties were adequate. PCR reagents were the same as above with PCR protocols of 94 °C for 1 min followed by 50 cycles of 94 °C for 45 s, 49-62 °C for 45 s, 72 °C for 15 s (Table 4-2). Following this, the primers were tested against 183 non-targets (Table 3-2) for cross reactivity and no amplification was observed. In addition, all primers were target tested against specimens of the respective stink bugs collected from the field with 100% amplification success.

4.3.4 Statistical Analysis

The proportion of predators testing positive for stink bugs was arc-sine square root transformed for heterogeneity of variance and analyzed by a ANOVA using a generalized linear model in SAS (SAS Institute, Cary, North Carolina, USA). The factors in this analysis were prey availability, week, and crop type. For 2011, a ANOVA was run for all predators combined. For 2012, one ANOVA was run for all predators combined and one was run for each of three focal predators. Differences among the locations and crop types were determined by using Tukey's Honest Significant Difference (HSD) tests.

Prey availability was the total number of stink bugs, adults and nymphs combined, of each species collected per field per date (200 sweeps). This number was used to represent the potential prey available to the generalist predators. It is used as a proxy for population levels as we did not have information on egg masses, which are the likely prey item for most of our screened predators. Differences between the means for different stink bug species was determined using a paired t-test in SAS with comparisons between *N. viridula* and *C. hilaris* and *N. viridula* and *E. servus*.

4.4 Results

4.4.1 Stink Bug Predation

A total of 2805 predators were tested for predation on stink bugs (Table 4-1). Seventeen of 1277 predators tested positive for *N. viridula* in 2011 and 72 of 1528 predators were positive for *N. viridula* in 2012. 1729 predators were tested for *E. servus*, with four individuals testing positive (*Coccinella septempunctata, Zelus* sp., *Geocoris* sp., and *Orius* sp.). 2133 predators were tested for *C. hilaris*, with 2 individuals testing positive (*Nabis* sp. and *Oxyopes* sp.). Overall predation on *E. servus* and *C. hilaris* was negligible and so they were excluded from all other analyses.

4.4.2 Predation on *N. viridula*

The following predators tested positive for *N. viridula*: *Coleomegilla maculata, Geocoris spp., Orius spp., Notoxus monodon, Nabis spp.,* and *Oxyopes spp*. In 2012, only groups that had positives from 2011 were repeated (Table 4-1). Predation in 2011 was very low for all predators with large sample sizes (Fig. 4-1) so predation by individual predators was only analyzed for 2012: *Geocoris spp.* (3.5%, 20/564), *Orius spp.* (9.8%, 39/399), and *Notoxus spp.* (4.7%, 13/279) (Fig. 4-2).

When all predators were combined in 2011 (overall model: $F_{36,12} = 3.17$, $p = 0.02$) there was a significant interaction of week and crop ($F_{11, 12} = 4.00$, $p = 0.01$) (Fig. 4-3, Table 4-3). There was higher predation on *N. viridula* in Bt-cotton compared to maturity group 7 soybean (Tukey's HSD, $P < 0.05$) (Fig. 4-3). There was a main effect of location on predation ($F_{11, 12} = 3.96$, $p = 0.05$) although no individual differences were detected in the Tukey's HSD.

In 2012, there were no significant effects on predation (overall model $F_{78, 29} = 0.95$, $p = 0.58$) (Table 4-4). Two of the predator groups, *Nabis spp.* and *Oxyopes spp*., had very low gut-content positives overall and *C. maculata* had zero gut-content positives in 2012, so these taxa were not analyzed (Table 4-1).

In 2012, the overall ANOVA for *Geocoris spp.* predation on *N. viridula* was not significant (overall model $F_{77, 19} = 1.73$, $p = 0.09$). There was however, a significant interaction of week and location ($F_{20, 19} = 3.17$, $p = 0.007$) on *Geocoris* spp. predation on *N. viridula* (Table 4-5) (Fig 4-2). Neither the overall ANOVA for *N. monodon* predation on *N. viridula* ($F_{18, 40} = 1.15$, $p = 0.34$) (Table 4-6) nor the ANOVA for *Orius* spp. predation on *N. viridula* was significant ($F_{18, 22} = 1.02$, $p = 0.48$) (Table 4-7).

4.4.3 Pest population numbers

In both years, the population numbers of *N. viridula* were higher than *C. hilaris* (2011: $t = 3.17$, $df = 127$, $Pr > |t| = 0.0019$; 2012: $t = 7.32$, $df = 243$, $Pr > |t| = <.0001$) and *E. servus* (2011: $t = 3.58$, $df = 127$, $Pr > |t| = 0.0005$; 2012: $t = 6.69$, $df = 243$, $Pr > |t| = <.0001$) (Table 4-8).

4.5 Discussion

Of the three stink bug species tested, *N. viridula* was the main prey item for generalist predators in our study. *N. viridula* also consistently had higher population levels in both years compared to the other species. This same trend has been observed in other studies in soybean (McPherson et al. 1979) and cotton (personal observation in (Greene et al. 1999)). Finding several different predators that consume *N. viridula* agreed with previous studies on stink bug predation (Ragsdale et al. 1981, Stam et al. 1987, Olson and Ruberson 2012, Greenstone et al. 2014a, Tillman et al. 2015). Predators with diverse feeding modes (sucking and chewing) consuming *N. viridula* may be promising for integrated pest management schemes in cotton and soybeans.

Our study uncovered several predators with diverse feeding modes (sucking: *Orius spp., Geocoris spp.,* and *Nabis spp.*; chewing: *N. monodon, Oxyopes spp.,* and *C. maculata*) that were consuming *N. viridula*. Although there is considerable observational and molecular evidence for which predators consume stink bugs, the studies vary as to the impact of these predators in agroecosystems. For predator groups where we had substantial sample sizes, the highest percentage testing positive was 9.8% and this was for *Orius spp.* in 2012. Our study ran over two years and we were never able to detect over ten percent of predators testing positive for any prey group. This low level of positive responses to stink bugs may reflect the availability of alternate prey (including other predators) for the generalist predators surveyed. The general lack of a change in frequency of positive detections in the predators when stink bug populations significantly increased late in the season (Figs. 4-1-4-3) also suggests that the predators were largely consuming other prey items in each system.

Our results contrast with two recent field studies employing molecular gut-content analysis to study predation on stink bugs and a related prey item, kudzu bug (*Megacopta cribraria*) in a cotton-soybean-peanut agroecosystem in the same region of the US (Greenstone et al. 2014a, Tillman et al. 2015). They found very high percentages of predators testing positive for kudzu bug and stink bugs. They also found many instances of individual predators simultaneously testing positive for kudzu bug and three species of stink bugs. *Geocoris spp.* were especially prone to this in their study, with 4% of *Geocoris punctipes* individuals testing positive for four pest species. In contrast, we did not uncover any instances of more than one prey item being detectable in a single predator and we had much lower gut-content positives, suggesting possible differences in assay sensitivity. There were also major differences in procedures used. These studies were done in a single location over a one-month period with samples collected in narrow experimental soybean strips adjacent to cotton and the first and second rows of the cotton plots (Greenstone et al. 2014a, Tillman et al. 2015). Further, one of their treatments contained buckwheat, which is known to provide nectar to generalist predators, especially *Geocoris spp.* (De Lima and Leigh 1984). In contrast, there were no nectar sources adjacent to our sampling sites and, we sampled at least 5 meters away from the field edges over 8-12 weeks at three well-separated locations, and replicated over two years. These procedural differences also could have contributed to some of the differences in our findings.

The contrast between other studies on stink bug predation and ours is not necessarily surprising as two of the three focal predators uncovered in this study, *Orius spp.*, and *Geocoris spp.* have been examined in several studies employing molecular gut-

content analysis in open field conditions and estimates of their gut-content positives are quite variable. Hagler and Blackmer (2013) tested *Geocoris spp*. collected in sweep nets for predation on three different prey items and found that the percent testing positive for any prey item varied from year to year. For example, in 2007, 15% of *Geocoris spp*. (N = 215) were found to be preying on *Bemisia tabaci*, whereas in 2008 (N = 160), 46% were positive for *B. tabaci*. An opposite trend was observed for *Lygus spp.*, with 35% of *Geocoris spp*. testing positive for them in 2007 and 4% testing positive for *Lygus spp*. in 2008.

Additionally, in a study investigating predation of the soybean aphid (*Aphis glycines*) variability was found with *Orius insidiosus*, with 13.4% of adults and 25% of immatures being gut-content positive for *A. glycines* (Harwood et al. 2009). The same trend was found with the other prey item they tested, *Neohydatothrips variabilis*, with 21.7% of adult *O. insidiosus* and 5.0% and immatures positive for *N. variabilis* (Harwood et al. 2009). A study on the same predators and prey, but not separated by life stage, found that *O. insidiosus* preyed upon *A. glycines* and *N. variabilis*, 65% and 35% of the time, respectively (Harwood et al. 2007b). Even within the same system, these generalist predators can vary greatly in their gut-content positives for the same prey items.

The differences in molecular techniques between studies could help explain the disparity in the frequency of the same species testing positive for predation. We screened our primers against 183 non-targets from 12 orders and 78 families (Table 3-3). This is in contrast to the non-target testing in other studies with 57 non-targets from 4 orders and 7 families (Greenstone et al. 2014a) and 83 non-targets from 3 orders and 7 families (Tillman et al. 2015). Cross amplification of primers can occur across very disparate taxa

(Chapman et al. 2013) emphasizing the need for strenuous non-target testing. Therefore, differences in primer design among studies may have contributed to differential gut-content amplification.

The variance in the frequency of a predator species testing positive for the same prey species from year to year, and the potential influence of different densities of available alternative prey on species interactions (Harwood et al. 2007b, Harwood et al. 2009, Hagler and Blackmer 2013) suggests that the effectiveness of generalist predators for controlling stink bug pests may need to be viewed on a case by case basis. In addition, our study highlights the need for replication studies. Our results contrast with previous studies occurring in the same crops in similar locations (Greenstone et al. 2014a, Tillman et al. 2015). The present study pinpointed several generalist predators that were consuming stink bug pests (*Geocoris spp.*, *N. monodon* 0 *Orius spp.*) and they had a low rate of testing positive for these pests. Before implementing a biological control scheme in a specific agroecosystem, it is important to know which natural enemies are having an impact on the focal pests. It is essential, therefore, that experiments exploring natural enemies on pests be replicated so we can optimize an integrated pest management scheme in cotton and soybean agroecosystems. A better understanding of the foraging behavior of these predators when a complex cocktail of prey species and densities are available would be needed to be able to predict their biological control potential in relation to a focal pest. And given the wide host ranges of stink bugs and the dispersal abilities of these pests, it's likely much more important to consider landscape-level biological control rather than simply focusing on crop fields.

Table 4-1. List of all predator taxa tested, with numbers testing positive in PCR testing. References contain observation evidence justifying inclusion of a given predator taxon.

Order	Family	Species/Group	2011		2012		Reference
			PCR (+) of *N. viridula*	PCR Tested for *N. viridula*	PCR (+) of *N. viridula*	PCR Tested for *N. viridula*	
Araneae	Anaphyanidae		0	3			
	Araneidae		0	6			(Ragsdale et al. 1981)
	Linyphiidae		0	10			
	Lycosidae		0	1			(Ehler 2002)
	Lycosidae	*Pardosa sp.*	0	12			
	Oxyopidae		0	12			
	Oxyopidae	*Oxyopes salticus*	1	163	0	174	(Ragsdale et al. 1981, Ehler 2002)
	Oxyopidae	*Peucetia virudans*	0	22	0	1	
	Salticidae		0	51			(Ragsdale et al. 1981)
	Salticidae	*Hentzia sp.*	0	10			
	Salticidae	*Sitticus sp.*	0	14	0	1	
	Tetragnathidae		0	2	0	2	(Ehler 2002)
	Theridiidae	*Latrodectus sp.*	0	1			
	Thomisidae		0	61	0	6	
Blattodea	Blattellidae	*Blattella asahinai*	0	24			[1](Pfannenstiel et al. 2008)
Coleoptera	Anthicidae	*Notoxus monodon*	3	79	13	279	[2](Stam et al. 1987, Ehler 2002)
	Carabidae		0	1			

Table 4-1 (continued)

Order	Family	Species/Group	2011 PCR (+) of *N. viridula*	2012 PCR Tested for *N. viridula*	PCR (+) of *N. viridula*	PCR Tested for *N. viridula*	Reference
	Coccinellidae	*Coccinella septempunctata*			0	5	(Ehler 2002, Tillman 2011)
	Coccinellidae	*Coleomegilla maculata*	3	9	0	87	(Ragsdale et al. 1981, Tillman 2011)
	Coccinellidae	*Harmonia axyridis*	0	15	0	10	(Ehler 2002, Tillman 2011)
Dermaptera	Forficulidae	*Doru taeniataum*	0	1			[1](Fenoglio and Trumper 2007)
	Labiduridae	*Labidura riparia*	0	13			[1](Fenoglio and Trumper 2007)
Hemiptera	Anthocoridae	*Orius spp.*	4	114	39	399	(Ragsdale et al. 1981, Tillman 2011)
	Labiduridae	*Labidura riparia*	0	13			[1](Fenoglio and Trumper 2007)
Hemiptera	Anthocoridae	*Orius spp.*	4	114	39	399	(Ragsdale et al. 1981, Tillman 2011)
	Coreidae	*Leptoglossus phyllopus*	0	2			
	Geocoridae	*Geocoris spp.*	5	258	20	564	(Ragsdale et al. 1981, Ehler 2002, Tillman 2011)
	Nabidae	*Nabis sp.*	1	142			(Ragsdale et al. 1981, Stam et al. 1987, Ehler 2002)
	Pentatomidae	*Podisus maculiventris*	0	21			(Ragsdale et al. 1981, Tillman 2011)
	Reduviidae	*Sinea spp.*	0	37			(Stam et al. 1987)
	Reduviidae	*Zelus spp.*	0	25			(Ehler 2002)

Table 4-1 (continued)

Order	Family	Species/Group	2011 PCR (+) of *N. viridula*	2012 PCR Tested for *N. viridula*	PCR (+) of *N. viridula*	PCR Tested for *N. viridula*	Reference
Orthoptera	Tettigoniidae		0	1			
Neuroptera	Chrysopidae	*Chrysoperla rufilabris*	0	12			(Stam et al 1987; [3]Ehler 2002)
	Hemerobiidae	*Micromus sp.*	0	1			

[1]Lepidopteran egg predators

[2]Beetle from the family Anthicidae

[3]Observation during laboratory feeding trials

Table 4-2. Primer names and sequences for taxa tested for consumption by predators.

Taxon	Primer Sequence	Amplicon size (bp)	Annealing Temp (°C)	Reference
N. viridula	NV-334F: 5'-TTTTTATTATTTATTTGGGTTG-3 NV-566R: 5'-GTCGAACAGACCTAGAAC-3'	245	53	Designed herein
E. servus	ES-43F: 5'-GTCTGATGTTATTTATATCAGATTTAA-3' ES-295R: -5'-AATAAATATTAACAATTTAACCAAAAC-3'	277	49	Designed herein
C. hilaris	AH-276F: 5'-AGACCCTATAGAATTTTATTTTAAAG-3' AH-390R: 5'-CCTAAAAATAATTATATTTAAACC-3'	146	53	(Penn et al. 2017)

Table 4-3. ANOVA table predation by all predators combined on *N. viridula* in 2011. Sum of Squares for factors are Type III. Crop types are maturity group 5, soybean maturity group 6.9, and Bt cotton. Prey availability is the total number of *N. viridula* (adults and nymphs) collected via sweep net. Sampling weeks were 29 July – 7 October 2011.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	36	0.68479234	0.01902201	3.17	0.02
Error	12	0.07206651	0.00600554		
Corrected Total	48	0.75685886			
Location	2	0.04753257	0.02376629	3.96	0.05
Crop	2	0.13571826	0.06785913	11.30	0.002
Week	7	0.23669475	0.03381354	5.63	0.005
Prey availability	1	0.02417603	0.02417603	4.03	0.07
Location*Crop	4	0.01641453	0.00410363	0.68	0.62
Location*Week	9	0.05413316	0.00601480	1.00	0.49
Crop*Week	11	0.26438236	0.02403476	4.00	0.01

Table 4-4. ANOVA table predation by all predators combined on *N. viridula* in 2012. Sum of Squares for factors are Type III. Crop types are maturity group 5, soybean maturity group 6.9, Bt cotton, and non-Bt cotton. Prey availability is the total number of *N. viridula* (adults and nymphs) collected via sweep net. Sampling weeks were 12 July – 11 October 2012.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	78	3.75011609	0.04807841	0.95	0.58
Error	29	1.46312203	0.05045248		
Corrected Total	107	5.21323812			
Location	2	0.01326135	0.00663068	0.13	0.88
Crop	3	0.04968397	0.01656132	0.33	0.80
Week	12	0.85314837	0.07109570	1.41	0.22
Prey availability	1	0.08084322	0.08084322	1.60	0.22
Location*Crop	4	0.32427806	0.08106951	1.61	0.20
Location*Week	22	1.15980375	0.05271835	1.04	0.45
Crop*Week	33	1.34005144	0.04060762	0.80	0.73

Table 4-5. Two-way ANOVA table *Geocoris spp.* predation on *N. viridula* in 2012. Sum of Squares for factors are Type III. Crop types are maturity group 5, soybean maturity group 6.9, Bt cotton, and non-Bt cotton. Prey availability is the total number of *N. viridula* (adults and nymphs) collected via sweep net. Sampling weeks were 12 July – 11 October 2012.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	77	4.51752653	0.05866918	1.73	0.09
Error	19	0.64378456	0.03388340		
Corrected Total	96	5.16131109			
Location	2	0.08257281	0.04128640	1.22	0.32
Crop	3	0.06403806	0.02134602	0.63	0.60
Week	12	1.67271046	0.13939254	4.11	0.003
Prey availability	1	0.01386715	0.01386715	0.41	0.53
Location*Crop	4	0.05293233	0.01323308	0.39	0.81
Location*Week	20	2.15099825	0.10754991	3.17	0.007
Crop*Week	32	0.97113854	0.03034808	0.90	0.62

Table 4-6. Two-way ANOVA table *Notoxus spp.* predation on *N. viridula* in 2012. Sum of Squares for factors are Type III. There were not enough degrees of freedom for all the interactions. Crop types are maturity group 5, soybean maturity group 6.9, Bt cotton, and non-Bt cotton. Prey availability is the total number of *N. viridula* (adults and nymphs) collected via sweep net. Sampling weeks were 12 July – 11 October 2012.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	18	1.14406684	0.06355927	1.15	0.34
Error	40	2.21035389	0.05525885		
Corrected Total	58	3.35442073			
Location	2	0.07096084	0.03548042	0.64	0.53
Crop	3	0.37531126	0.12510375	2.26	0.1
Week	12	0.67877007	0.05656417	1.02	0.45
Prey availability	1	0.05155230	0.05155230	0.93	0.34
Location*Crop	0	0	0	0	0
Location*Week	0	0	0	0	0
Crop*Week	0	0	0	0	0

Table 4-7. Two-way ANOVA table *Orius spp.* predation on *N. viridula* in 2012. Sum of Squares for factors are Type III. There were not enough degrees of freedom for the interactions. Crop types are maturity group 5, soybean maturity group 6.9, Bt cotton, and non-Bt cotton. Prey availability is the total number of *N. viridula* (adults and nymphs) collected via sweep net. Sampling weeks were 12 July – 11 October 2012.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	18	1.29868768	0.07214932	1.02	0.48
Error	22	1.55441378	0.07065517		
Corrected Total	40	2.85310145			
Location	2	0.10823481	0.05411741	0.77	0.48
Crop	3	0.04815640	0.01605213	0.23	0.88
Week	12	0.84790309	0.07065859	1.00	0.48
Prey availability	1	0.03579425	0.03579425	0.51	0.48
Location*Crop	0	0	0	0	0
Location*Week	0	0	0	0	0
Crop*Week	0	0	0	0	0

Table 4-8. Mean stink bugs collected by 100 sweeps by sweep net across the season

Stink Bug Species	2011 Mean ± SE	2012 Mean ± SE
N. viridula	3.8 ± 0.71	2.6 ± 0.35
C. hilaris	1.8 ± 0.27	0.1 ± 0.04
E. servus	1.7 ± 0.24	0.7 ± 0.11

Figure 4-1. Mean (±SE) number *N. viridula* per 100 sweeps and the proportion *Geocoris spp., Orius spp,* and *Notoxus monodon* testing positive for *N. viridula* DNA by sampling week, 29 July – 7 October 2011.

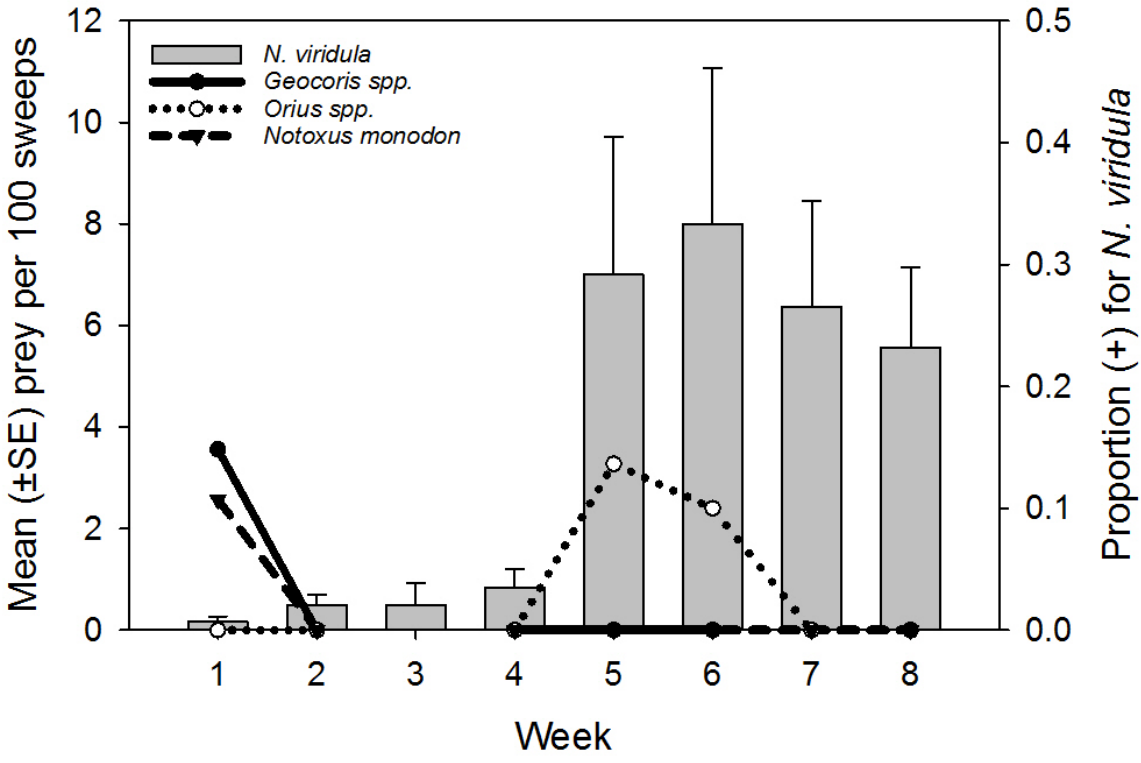


Figure 4-2. Mean (±SE) number of *N. viridula* per 100 sweeps and the proportion *Geocoris spp*, *Orius spp*, and *N. monodon.* testing positive for *N. viridula* DNA by sampling week, 12 July – 11 October 2012.

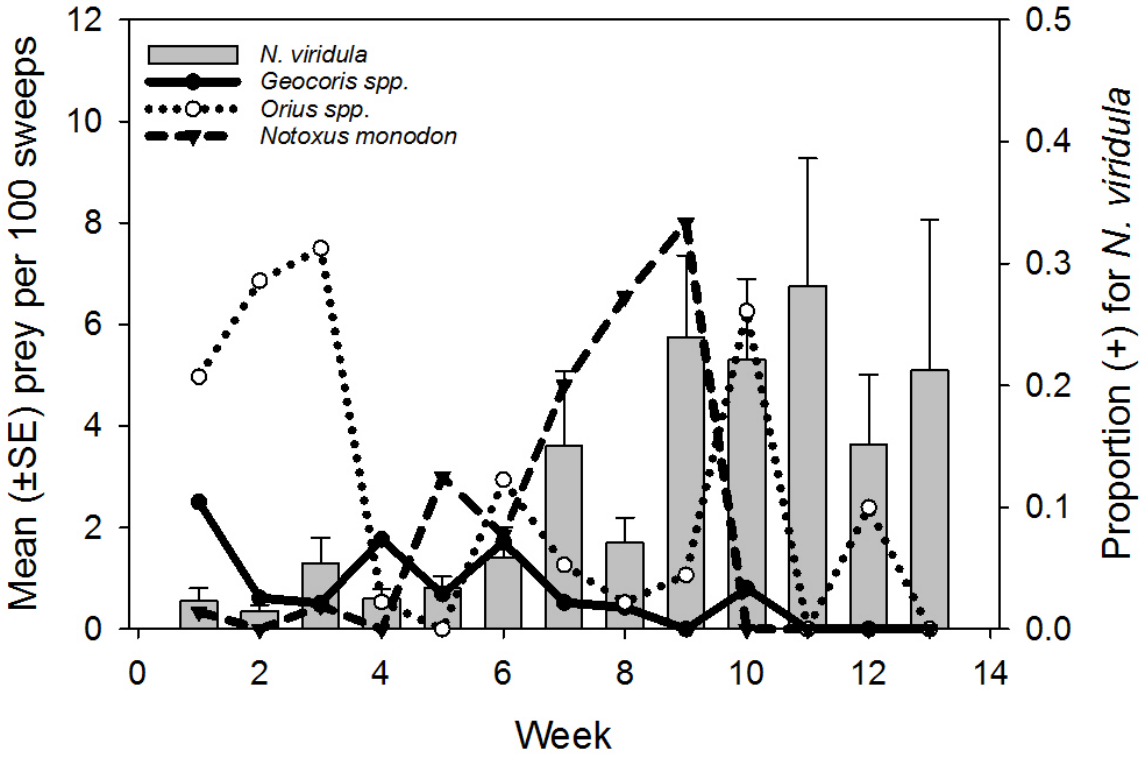


Figure 4-3. Proportion of predators testing positive for *N. viridula* DNA by crop and sampling week, 29 July – 7 October 2011. Predators are *Coleomegilla maculata*, *Geocoris spp.*, *Orius spp.*, *Notoxus monodon*, *Nabis spp.*, and *Oxyopes spp.*

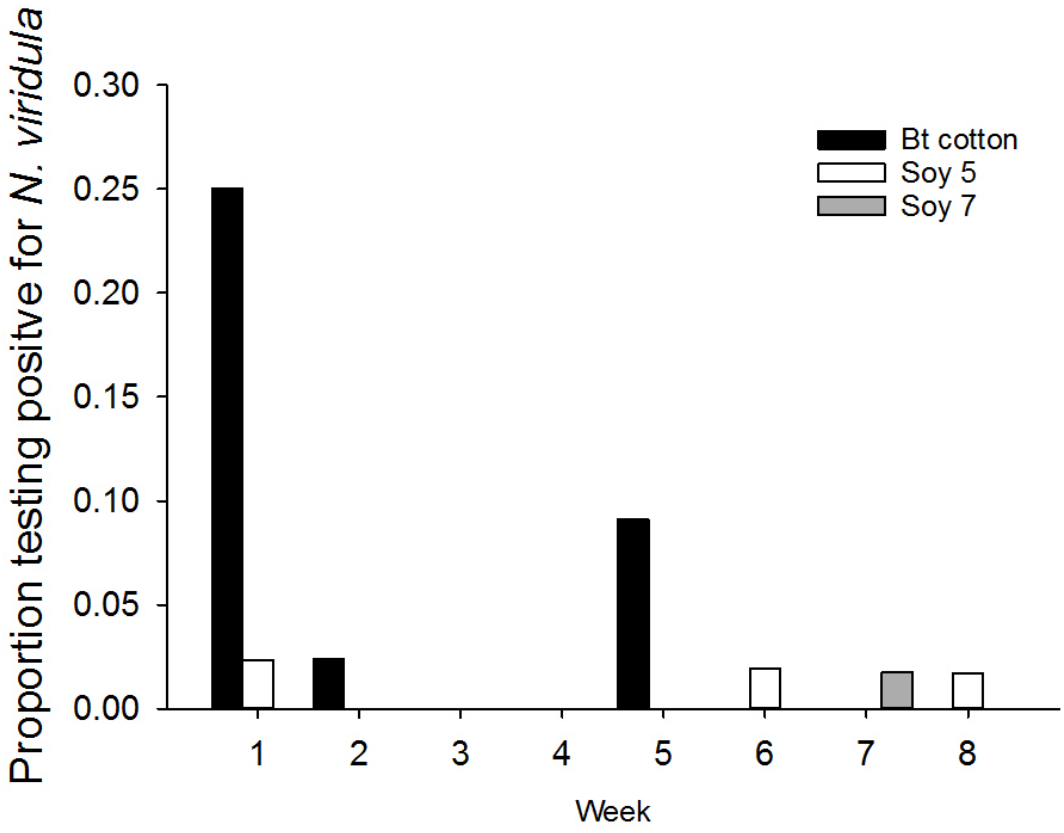
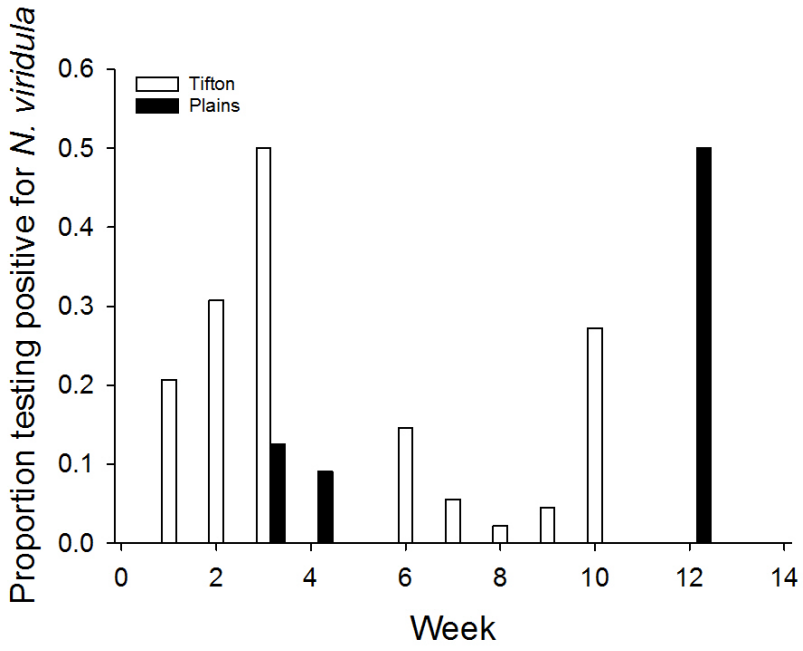


Figure 4-4. Proportion of predators testing positive for *N. viridula* DNA by crop and sampling week, 12 July – 11 October 2012. Predators are *Coleomegilla maculata*, *Geocoris spp.*, *Orius spp.*, *Notoxus monodon*, *Nabis spp.*, and *Oxyopes spp.*



Chapter 5: Detection of the invasive kudzu bug (*Megacopta cribraria*) beyond its invaded front through molecular gut-content analysis

5.1 Summary

The kudzu bug, *Megacopta cribraria* (Hemiptera: Plataspidae) was discovered in the United States in 2009 in northeast Georgia, has invaded thirteen states and is a significant pest in soybean. *Megacopta cribraria* is also a beneficial herbivore of kudzu with a large impact on stands of kudzu, complicating the status of this invasive insect. Predators of *M. cribraria* are poorly understood and in need of investigation. As field observation of predation is difficult, molecular gut-content analysis offers an alternative to traditional approaches. Consequently, species-specific 16S molecular markers were designed and used to discern the gut-contents of potential predators of *M. cribraria*. Over 2300 predators were collected during two growing seasons in cotton and soybean in southern Georgia in 2011 and 2012, screened for *M. cribraria* DNA and trophic linkages were analyzed relative to prey availability, crop type and field location. Our results indicate *M. cribraria* was consumed primarily by *Geocoris spp*. We detected *M. cribraria*, in a previously undocumented county in Georgia, a year before it was detected by standard visual or mechanical trapping methodologies. Implications of using molecular techniques to track invasive species, especially early in their spread are discussed.

5.2 Introduction

Biotic invasions occur when organisms are transported to new areas where they proliferate, spread and persist (Elton 1958). Since the late 1950s, invasion by non-native species has been recognized as a critical problem in our highly mobile and connected

world (Elton 1958, Vitousek 1996, Mack et al. 2000, Paini et al. 2016). These organisms can do extreme damage in their new ranges including displacing native species, restructuring habitats and ecosystems, and even destroying certain habitats. For example, *Littorina littorea* (L.) has restructured intertidal ecosystems (Bertness 1984); an invasive moss, *Campylopus introflexus* (Hedw.) Brid., has altered arthropod assemblages in acidic coastal dunes (Schirmel et al. 2011); and gypsy moth (*Lymantria dispar*) populations can be so large that they alter soil organic matter dynamics on the forest floor with nutrient pulses from their feces, dead bodies and unconsumed green foliage (Lovett et al. 2006)). In addition to damaging natural habitats, invasive species may have significant effects on agroecosystems. It is estimated that overall damages to natural and managed ecosystems and control of invasive species can have costs of up to $120 billion/year in the US (Pimentel et al. 2005). It is further estimated that the United States spends about $7.4 billion dollars on pesticides, for plants and animals, much of which goes to control non-indigenous pests (U.S. Congress 1993).

When invasive species move into an area, they can cause major shifts in the existing food web. Invasive purple loosestrife (*Lythrum salicaria* L.) can recruit additional pollinators and dragonflies, increasing larval dragonfly abundance and also changing zooplankton richness (Burkle et al. 2012). Additionally, the opossum shrimp (*Mysis diluviana* Audzijonyte & Väinölä) changes the fish community in a freshwater lake with affects cascading to the top predators in the system (Ellis et al. 2011). Overall, invasive species could displace native prey and become an abundant prey resource for native predators (Carlsson et al. 2009). The zebra mussel (*Dreissena polymorpha* (Pallas)) has numerous effects on habitats it invades, including shifts in food webs when

predatory amphipods benefit from zebra mussel biodeposits (Gergs et al. 2011) and inducing native fish to consume zebra mussel over the native mussels, possibly resulting in lower parasite loads in native fish (Locke et al. 2014). Native predators often respond to large invasions of prey items into their habitats which can dramatically change the food available to them (Carlsson et al. 2009). Pintor and Byers (2015) conducted a meta-analysis and found that predators could benefit from non-native prey as a supplemental resource. Additionally, predators can exploit recent invaders into agricultural settings, as observed with the invasive brown marmorated stink bug (*Halyomorpha halys* (Hemiptera: Pentatomidae) (Morrison et al. 2016).

In 2009, *Megacopta cribraria* (kudzu bug) (Hemiptera: Plataspidae) was discovered in the United States in northeast Georgia (Eger et al. 2010). This native to Asia has spread quickly in North America, with an estimated range expansion from 7,050 km^2 in 2009 to 98,816 km^2 in 2010, and subsequent spread to 13 states (Gardner et al. 2013, KudzuBug 2017). These bugs are strong fliers and are known to hitchhike on vehicles (Gardner et al. 2013). *Megacopta cribraria* was found in large numbers on houses near kudzu, *Pueraria montana* (Lour.) Merr. (Fabales: Fabaceae) where it had developed and then moved into houses to overwinter (Eger et al. 2010).

Megacopta cribraria can develop on several plants species including soybean, kudzu, and other legumes, although they lay most of their egg masses on soybean and kudzu in green house experiments (Medal et al. 2016). Although *M. cribraria* was found on several legume species in field settings, complete development occurred only on kudzu and soybean (Zhang et al. 2012). *Megacopta cribraria* can be both a beneficial and a pest species as their preferred plants are kudzu, one of the most serious invasive weeds

in the United States (Myers and Bazely 2003), and soybean, a valued agricultural crop (e.g. Zhang et al. 2012, Ruberson et al. 2013, Medal et al. 2016). *Megacopta cribraria* has been shown to decrease kudzu growth by 33% in controlled field plots (Zhang et al. 2012), and has removed 80% of kudzu aboveground biomass over a period of three years in open field observations (Gardner and Olson 2016). However, it can also significantly reduce soybean yield (Seiter et al. 2013). In addition to soybean, *M. cribraria* has been observed on cotton, but this is probably a non-host on which *M. cribraria* rest or congregate (Gardner et al. 2013).

In less than four years, from October 2009-July 2012, *M. cribraria* spread from nine counties in Georgia to 392 counties in eight states (Gardner et al. 2013). During this swift invasion, a project using molecular gut-content analysis to study predation on stink bugs in soybean and cotton in Georgia, U.S.A., was underway (Chapter 4). A variety of generalist predators were collected and screened for predation on southern green stink bug, *Nezara viridula* (L.), green stink bug, *Chinavia. hilaris* (Say) and brown stink bug, *Euschistus servus* (Say). There were three locations where sampling was conducted, two of them had documented *M. cribraria* invasion in 2011, and one of the locations did not have *M. cribraria* invasion until 2012. We investigated predation on the invasive *M. cribraria* in soybean and cotton at the three locations in both years to determine which predators were eating *M. cribraria* and if we could detect predation on *M. cribraria* in areas where researchers had not yet documented the kudzu bug invasion using standard sampling techniques, such as sweep net sampling and visual observations (Gardner et al. 2013).

5.3 Materials and Methods

5.3.1 Field Sampling

Field sampling took place in soybean-cotton farms from July through October 2011 and 2012 in three locations in southwestern Georgia, USA. The locations were Belflower Farm, Tifton, GA (Tift Co.) (N31° 30.434 W083° 33.430), the Southwest Research and Education Center, Plains, GA (Sumter Co) (N32° 03.589 W84° 36.691) where *M. cribraria* was detected in 2011, and the Attapulgus Research and Education Center, Attapulgus, GA (Decatur Co) (N30°76.254 W84° 48.488) where *M. cribraria* was not detected until 2012 (KudzuBug 2017). In 2011, three crops at each location were sampled: Bt-cotton (DP1034B2RF), soybeans MG5 (maturity group 5) (Agsouth Genetics 568RR) and soybeans MG6.9 (maturity group 6.9) (Asgrow AG6931RR) (Monsanto Co, St. Louis, MO, USA). In 2012, four fields were sampled: Bt-cotton, non-Bt cotton, MG5 soybeans and MG7 soybeans. Aldicarb (Bayer CropScience Leverkusen, Germany), was applied in furrow at planting (3.93 kg/ha (3.5 lbs/acre)) for thrips control (Grey et al. 2006). No other insecticides were applied. Each year was analyzed separately because of the addition of non-Bt cotton in 2012.

5.3.2 Arthropod Sampling

Predators and *M. cribraria* were collected biweekly (2011: 29 July- 7 October, 2012: 12 July – 11 October) via a 31 cm diameter sweep net with 100 sweeps per sample, two samples per field which were pooled for subsequent analyses. Each sample was taken along two different rows separated from one another by six rows. Sweeping was initiated five meters into the crop and along rows at least five rows from the plot edge to reduce edge effects. Different rows were sampled on each sample date to prevent prolonged

disruption of sampled rows. All arthropods were counted with predators and *M. cribraria* immediately separated and placed in sterile 1.5 mL microcentrifuge tubes filled with 95% ethanol. Identifications were to the lowest taxonomic level possible and specimens were then frozen at -20°C until subsequent DNA analysis.

5.3.3 Molecular Gut-content Analysis

Total DNA was extracted from all predatory specimens using DNeasy Blood and Tissue Kits© (Qiagen Inc., Valencia, CA, USA) following standard animal tissue protocols. For primer design, stink bug legs were removed and extracted. For molecular gut-content analysis, all predators were crushed and whole body extracted.

For primer design, we amplified 16S sequences using general primers; 16Sbr-H and 16Sar-L (Palumi et al. 1991). Polymerase chain reactions (PCR) (25 µL) consisted of 1X Takara buffer (Takara Bio Inc., Shiga, Japan), 0.2 mM of each dNTP, 0.2 mM of each primer, 1.25 U Takara Ex Taq™ and template DNA (1-2 µL of total DNA). PCRs were carried out in Bio-Rad PTC-200 and C1000 thermal cyclers (Bio-Rad Laboratories, Hercules, CA, USA). The PCR protocol was 94 °C for 1 min followed by 50 cycles of 94 °C for 45 s, 63 °C for 45 s, 72 °C for 45 s and a final extension of 72 °C for 5 min. PCRs included a positive and negative control. Following amplification, the bands were visualized on 2% SeaKem agarose (Lonza, Rockland, Maine, USA) pre-stained with GelRed nucleic acid gel stain (1X; Biotium, Hayward, California, USA). The PCR product was purified and sequenced at AGTC (University of Kentucky, Lexington, KY, USA).

Sequences were editing using Geneious© (Biomatters Ltd, Auckland, New Zealand) and aligned using MUSCLE (Edgar 2004). We designed primers by visually

inspecting the sequences using BioEdit 7.0.0 (Isis Pharmaceuticals Inc., Carlsbad, CA, USA) and then using Primer3 (Rozen and Skaletsky 2000) to determine whether the primer properties were adequate. PCR reagents were the same as above with 94 °C for 1 min followed by 50 cycles of 94 °C for 45 s, 62 °C for 45 s, 72 °C for 15 s. The primers designed for amplification of *M. cribraria* are MC-288F (5'-CCCTATAGAAATTTACTCTATTTTTGGTG-3') and MC-454R (5'-GAAATTACGCTGTTATCCCTAAGGTAAA-3') producing a 193 bp amplicon. Following this, the primers were tested against 183 non-targets (Table 3-2) for cross reactivity and no amplification was observed. In addition, primers were target tested against specimens of *M. cribraria* collected from the field with 100% amplification success.

5.3.4 Statistical Analysis

The proportion of predators testing positive for *M. cribraria* was arc-sine square root transformed for normality and analyzed by ANOVA using a generalized linear model (GLM) in SAS (SAS Institute, Cary, North Carolina, USA). The factors in this analysis were prey availability, week, location, and crop type. Prey availability was the total number of *M. cribraria* collected per field per date (200 sweeps). This number was used to represent the potential prey available to the generalist predators. It is used as a proxy for population levels as we did not have information on egg masses, which are the likely prey item for most of our screened predators.

5.4 Results

In 2011, four predators tested positive for *M. cribraria* DNA in the Attapulgus location, even though *M. cribraria* was not detected from samples in this location. In

week 4 (26 Aug), 1/3 *Oxyopes spp.* were positive for *M. cribraria* predation in Bt cotton and 2/2 were positive for *M. cribraria* predation in maturity group 5 soybean. In week 7 (23 Sept.), 1/2 *Nabis spp.* were positive for *M. cribraria* predation (Table 5-1). *Geocoris spp*. were also positive for *M. cribraria* predation in the Plains and Tifton locations where kudzu bug had been previously detected (Fig. 5-1). A total of five *M. cribraria* were collected from all crops in 2011 in the Plains and Tifton locations. For all predator groups, combined, 2.3% of predators (4/175) were positive for *M. cribraria* predation in 2011 at the Attapulgus location, whereas 1.7% of predators (3/176) were positive for *M. cribraria* predation in 2012. There was no significant difference between the years in this location ($F_{1, 79} = 0.29$, $p = 0.59$) (Table 5-2).

Overall, 1.9% (16/836) of the predators tested were positive for *M. cribraria* in 2011. In 2012, 5.2% (78/1505) of the predators were positive for *M. cribraria* predation. The predator groups were *Geocoris spp.*, *Orius spp.*, *Nabis spp.*, *C.*, *N. monodon*, *Oxyopes spp.*, Linyphiidae, Thomisidae (Table 5-3).

Geocoris spp. had the highest percentage (3%) of gut-content positives in 2011. The overall ANOVA was not significant ($F_{33, 6} = 1.49$, $p = 0.33$) Neither location ($F_{2, 6} = 2.81$, $p = 0.14$), crop type ($F_{2, 6} = 3.01$, $p = 0.12$), nor week ($F_{6, 6} = 1.64$, $p = 0.28$) affected predation of *M. cribraria* (Table 5-4). *Geocoris spp*. also had the highest percentage (9.4%) of gut-content positive for *M. cribraria* predation in 2012 (overall ANOVA: $F_{87, 7} = 3.99$, $p = 0.03$). There was a significant effect of week ($F_{12, 7} = 4.97$, $p = 0.02$) (Fig. 5-2). There was no effect of location ($F_{2, 7} = 1.54$, $p = 0.28$) or crop ($F_{3, 7} = 1.34$, $p = 0.34$) on predation of *M. cribraria* (Table 5-5). All other predator groups had low positives for *M. cribraria* predation and were not analyzed (Fig. 5-2).

5.5 Discussion

Early detection of an invasive species is key to helping reduce its spread and harm (Pysek and Richardson 2010), whereby early detection may make control and eradication simpler (Simpson et al. 2009, Vander Zanden et al. 2010). One of the newest ways to detect invasive species is through the use of DNA based technologies (Jerde et al. 2011). Many of these studies have been conducted using environmental DNA (eDNA) and screening that for the presence of an invasive species (Jerde et al. 2011). This has been done extensively in aquatic systems (Harvey et al. 2009b, Jerde et al. 2011, Dejean et al. 2012, Clusa et al. 2016). Recently, this technique has been used in terrestrial systems with an assay developed to utilize eDNA to track another invasive terrestrial pentatomoid, *H. halys* (Valentin et al. 2016). We used DNA screening and detected *M. cribraria* in the guts of predators although we never detected *M. cribraria* in our field samples at Attapulgus in 2011. The percentage of predators screening positive for *M. cribraria* DNA was 2.3% in 2011 at the Attapulgus location, whereas 1.7% of predators were positive for *M. cribraria* predation in 2012 when a low density of *M. cribraria* (mean ± SE = 2.88 ± 0.58 bugs per 100 sweeps) were collected in our samples. Generalist predators often consume rare prey items at disproportionate rates (Athey et al. 2016). This is generally seen in the early season predation literature, but could also pertain to invasive species in the early introduction or colonization stage of their invasive spread. In addition, as our primers underwent extensive non-target testing, with 183 exemplars from twelve orders and 78 families, we are confident that we were not observing false positives. Additionally, it is unlikely that these predators were eating *M. cribraria* in already infested areas and then flying to our fields as there is a several country buffer

around the Attapulgus location where *M. cribraria* were not detected until 2012 (KudzuBug 2017).

Predation on *M. cribraria*, overall was low. Even with very large numbers of *M. cribraria* in the landscape, with up to 935 (± 410) per 100 sweeps in week 12 in 2012, *Geocoris spp.* which had the highest number of individuals testing positive, only had 9.4% positive indicators of *M. cribraria* predation. Much like previous studies on stink bugs and *M. cribraria*, we found a diverse suite of predators consuming *M. cribraria* (Chapter 4; (Greenstone et al. 2014a, Tillman et al. 2015)). These predators had diverse feeding modes; sucking: (*Orius spp.*, *Geocoris spp.*, and *Nabis spp.*; chewing: *N. monodon, Oxyopes spp.*, Linyphiidae, Thomisidae and *C. maculata*). Predators were consuming kudzu bug at an overall comparable rate to their consumption of the naturalized southern green stink bug (*Nezara viridula* (L.) in our experimental locations (Chapter 4). Our results indicate that generalist predators readily exploit a new invasive species, albeit at low levels at present and may be useful as a part of an IPM strategy over time.

To our knowledge, this is the first study to document the spread of an invasive species to a new location through molecular gut-content analysis. Screening for *M. cribraria* was done within the context of a study examining predation on several species of stink bugs (Chapter 4). We chose to additionally screen the predators for *M. cribraria*, as the invasion of this species was occurring at the time of our study. We therefore already had DNA extracted for predators in invaded and non-invaded locations allowing us examine predation on the invasive species along its invasion front. This technique may not be useful on a large scale, but recent techniques in biodiversity studies could be

utilized to study invasive species (Yu et al. 2012). Large scale trapping along an invasion front, followed by high throughput sequencing on homogenized samples (Yu et al. 2012) with specific primers used to amplify the target invasive should be a useful technique for invasive species detection. Use of specific primers would amplify rare targets without needing to design blocking primers. Each large sample, whether it was a sweep sample, malaise trap samples (Yu et al. 2012, Brandon-Mong et al. 2015, Moriniere et al. 2016) or soil samples (Yang et al. 2014, Andújar et al. 2015) could be combined because, unlike in our study, specific trophic interactions are not the goal, only detecting the presence of an invasive species.

The probability of detecting an invasive species can be quite low. For example, a study on an introduced water flea (*Cercopagis pengoi*) found that the probability of detecting, using traditional zooplankton sampling techniques, when it was at low population densities was very low (< 0.2). This was true even with sampling intensity 20 times higher than what is typically employed by plankton researchers. Detection was only possible when the population densities were high (Harvey et al. 2009a). In our study, we detected *M. cribraria* DNA in the guts of predators when sweep net sampling, the most common method for detecting *M. cribraria* in soybean and kudzu (Gardner et al. 2013), was not sufficient to detect it. This suggests that molecular methods have the potential to detect invasive species when they are in very low levels without intensive sampling.

Metabarcoding could be a powerful tool for detecting unknown invasive species because it does not require an a priori knowledge of the species present in a sample (Comtet et al. 2015). Metabarcoding samples could therefore be used to monitor many

invasive species at once. Additionally, methods could be developed that would combine general and specific primers and could assess the biodiversity in an area while simultaneously screening for the presence of a specific invasive species, such as kudzu bug. This would also allow amplification of degraded DNA, such as gut-contents (Piñol et al. 2014, Macías-Hernández et al. 2017), which in the case of our study was the only means of detection for *M. cribraria* in the location where invasion had not been documented.

However, metabarcoding could result in the amplification of DNA from the guts of highly mobile predators and might result in false positives for a given area. Predators testing positive for habitat-specific pests of neighboring crops have documented the movement of predators between crops (Greenstone et al. 2014a). Predators testing positive could overestimate the spread of an invasive species, but since it is important to have early detection, this could be an early warning that an invasive species is nearby (Comtet et al. 2015).

We detected an invasive species, kudzu bug, using molecular gut-content analysis in a previously undocumented county in Georgia, a year before it was detected by researchers using sweep net sampling. This research highlights the utility of molecular techniques to track invasive species, even with highly degraded DNA, like gut-contents. Employing next generation sequencing techniques for invasive species research could allow us to detect invasions early and possibly limit their spread and damage.

Table 5-1. Predators testing positive for *M. cribraria* in 2011. All predator groups except *Geocoris spp.* are shown. *Geocoris spp*. proportion testing positive is found in Figure 5-1.

Predator	Total (+)	Total tested	Location and crop	Week	*M. cribraria* present[1]	Number collected[2]
Nabis spp.	1	2	Attapulgus, soy 7	7	No	NA
Linyphiidae	1	2	Plains, soy 7	6	Yes	0
Thomisidae	1	2	Tifton, soy 7	4	Yes	0
	1	1	Plains, soy 7	6	Yes	0
Oxyopes spp.	1	3	Attapulgus, Bt cotton	4	No	NA
	2	2	Attapulgus, soy 5	4	No	NA

[1]This column denotes whether *M. cribraria* was detected in this location in 2011 via sweep net. (http://www.kudzubug.org/distribution-map/).

[2]This column denotes number of *M. cribraria* collected in this location and crop in the week that gut-content positives occurred.

Table 5-2. ANOVA table predation on *M. cribraria* by all predators combined in the Attapulgus location. Sum of Squares for factors are Type III. Prey availability is the total number of *M. cribraria* (adults and nymphs) collected via sweep net.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	2.61376539	0.07920501	1.09	0.37
Error	79	5.76224590	0.07293982		
Corrected Total	112	8.37601129			
Crop	2	0.29282500	0.14641250	2.01	0.14
Week	12	1.23489219	0.10290768	1.41	0.18
Year	1	0.02140887	0.02140887	0.29	0.59
Prey availability	1	0.00820004	0.00820004	0.11	0.74
Crop*Week	17	1.26694357	0.07452609	1.02	0.45

Table 5-3. List of all predator taxa tested, with numbers testing positive for *M. cribraria* in PCR testing

			2011			2012		
Order	Family	Species/Group	No. tested for *M. cribraria*	No. (+)	% PCR (+)	No. tested for *M. cribraria*	No. (+)	% PCR (+)
Araneae	Linyphiidae		10	1	10	0	0	0
	Oxyopidae	*Oxyopes spp.*	163	3	1.8	169	7	4.1
	Thomisidae		61	2	3.3	6	0	0
Coleoptera	Anthicidae	*Notoxus monodon*	79	0	0	279	3	1.1
	Coccinellidae	*Coleomegilla maculata*	9	0	0	87	4	4.6
Hemiptera	Anthocoridae	*Orius spp.*	114	0	0	399	11	2.8
	Geocoridae	*Geocoris spp.*	162	5	3	565	53	9.4
	Nabidae	*Nabis sp.*	142	1	0.7	0	0	0

Table 5-4. ANOVA table predation on *M. cribraria* by *Geocoris spp.* in 2011. Sum of Squares for factors are Type III.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	0.81046698	0.02455961	1.48	0.33
Error	6	0.09984832	0.01664139		
Corrected Total	39	0.91031529			
Location	2	0.09361131	0.04680565	2.81	0.14
Crop	2	0.10002910	0.05001455	3.01	0.12
Week	6	0.16368869	0.02728145	1.64	0.28
Location*Crop	4	0.04573168	0.01143292	0.69	0.63
Crop*Week	11	0.30805580	0.02800507	1.68	0.27
Location*Week	8	0.09935040	0.01241880	0.75	0.66

Table 5-5. ANOVA table predation on *M. cribraria* by *Geocoris spp.* in 2012. Sum of Squares for factors are Type III. Prey availability is the total number of *M. cribraria* (adults and nymphs) collected via sweep net.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	87	17.74360038	0.20394943	3.99	0.03
Error	7	0.35798442	0.05114063		
Corrected Total	94	18.10158480			
Location	2	0.15734265	0.07867132	1.54	0.28
Crop	3	0.20556671	0.06852224	1.34	0.34
Week	12	3.04929432	0.25410786	4.97	0.02
Prey availability	1	0.00289431	0.00289431	0.06	0.82
Location*Crop	4	0.07905485	0.01976371	0.39	0.81
Crop*Week	26	1.24894747	0.04803644	0.94	0.59
Location*Week	17	0.54471977	0.03204234	0.63	0.80
Prey availability*Week	8	0.11535247	0.01441906	0.28	0.95
Prey availability*Crop	2	0.06118845	0.03059423	0.60	0.58
Prey availability*Location	2	0.06862248	0.03431124	0.67	0.54

Figure 5-1. Mean (±SE) number of *Geocoris spp.* and *M. cribraria* (kudzu bug) per 100 sweeps and the proportion *Geocoris spp.* testing positive for *M. cribraria* DNA by sampling week (29 July – 7 October 2011).

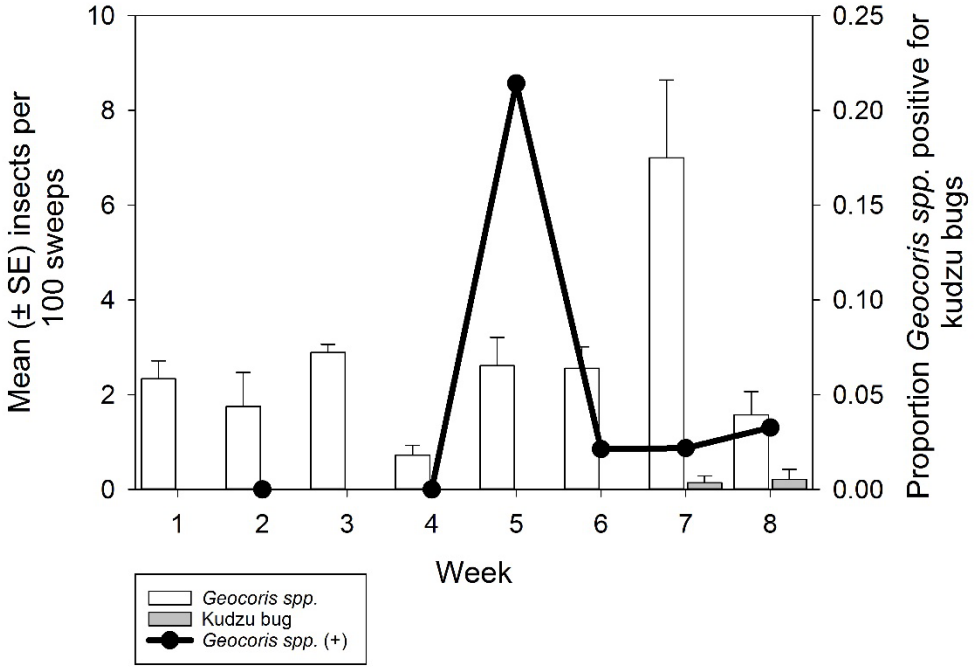
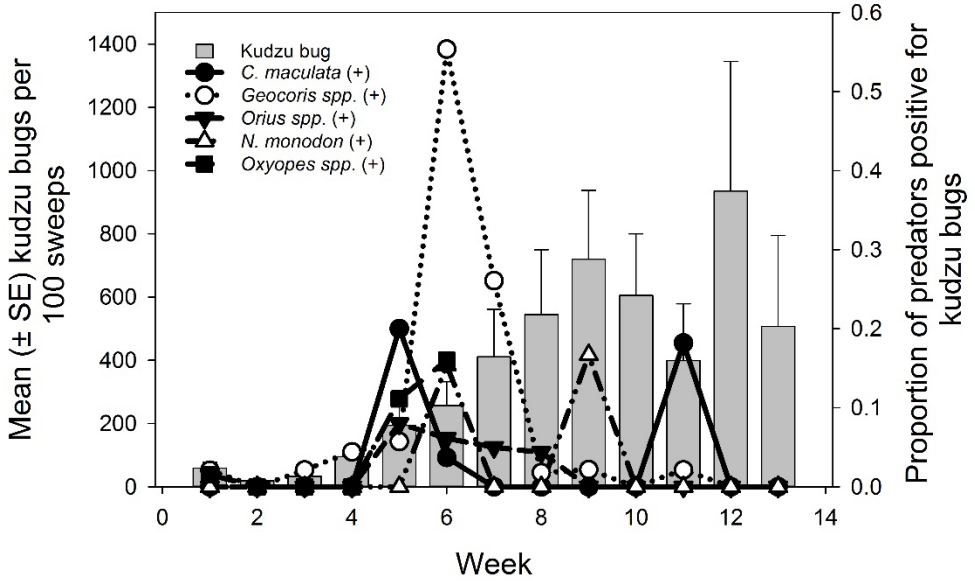


Figure 5-2. Mean (±SE) number of *M. cribraria* (kudzu bugs) per 100 sweeps and the proportion predators testing positive for *M. cribraria* DNA by sampling week. (12 July – 11 October 2012).



Chapter 6: Synthesis

In this dissertation, I clarified predator prey interactions in agroecosystems using molecular gut-content analysis combined with ecological experimentation. Contamination and false positives (Chapman et al. 2010, Greenstone et al. 2011, Greenstone et al. 2012, King et al. 2012) are of concern in molecular gut-content analysis, and each technique for collecting and preserving specimens for molecular work should be scrutinized to minimize experimental error. This may be especially true with food webs in agroecosystems where management techniques recommended to farmers may be costly. Ensuring that the results generated show generalist predators engaging in consumption of a focal pest and are not generating false positives is crucial. I showed that gut-content contamination is not present in fluid filled pit-fall traps and may allow this technique to be utilized for sampling of predators for molecular gut-content analysis. Fluid filled pitfall traps eliminate the risk of predation within the trap. Their use will also allow nocturnal and cryptic predators to be collected in a more efficient, less labor-intensive way without needing to collect each individually by hand. Additionally, I showed that storing specimens together in ethanol does not cause cross contamination and this may allow researchers to utilize mass sampling techniques, such as malaise traps for specimens destined for molecular gut-content analysis without risk of gut-content contamination. Mass sampling is more efficient than hand collecting of specimens, which is the current recommended best practice (King et al. 2008) and utilizing mass sampling techniques, such as wet pit-fall traps will allow researchers to expand the taxa used for food web characterization in molecular gut-content analysis. The future directions would be to undergo rigorous testing of other sampling techniques, such as malaise traps to

determine how long DNA is viable in the gut in this technique and if there is gut-content contamination using this tool. If not, this mass collecting technique could be utilized to collect specimens for gut-content analysis.

In addition to contamination, false positives may result from primer design. PCR is a very sensitive technique and well-designed primers should only amplify DNA from target species or group of species, depending on the goal of the project. While investigating the food webs of stink bugs in cotton and soybean, I obtained results that were in contrast to previous studies on stink bug food webs in the same agroecosystems (Greenstone et al. 2014a, Tillman et al. 2015). My non-target testing was more rigorous and suggests their very high percentage of predators screening positive for stink bug DNA may have been due, in part, to non-target amplification. Although I did not directly test their primers, the disparity in our results highlights the need for replicated studies when examining generalist predators for possible conservation biological control programs. In this case, future studies could compare non-target amplification of by specific primers that target the same species but were designed by different researchers. When investigating possible conservation biological control using specific natural enemies, more replication is needed before making management recommendations to growers.

Generalist predators can also have non-consumptive effects on pest species (Maupin and Riechert 2001, Preisser et al. 2005), and I sought to explore such effects in this dissertation. I combined molecular gut-content analysis with a field cage study to examine the relationship between a stink bug pest and a generalist predator. I detected no

predation within the cages, but I detected superfluous killing of stink bugs by spiders, only occurring in the treatments with high numbers of stink bugs. Combined, my results suggest that these spiders do not have significant impacts on stink bug populations, either through consumptive or non-consumptive effects. The evidence from my research suggests that an integrated pest management strategy for stink bug pests should not concentrate on generalist arthropod predators as their impacts on the populations may be negligible.

Most of this dissertation focused on the relationships between generalist predators and pests in the superfamily Pentatomoidea. Overall, the percentage of predators that screened positive for stink bugs and kudzu bugs was low which is in direct contrast to previous studies. I used molecular gut-content analysis to detect the presence of *M. cribraria* in areas where they had not been detected by monitoring tools like sweep nets. This is the first instance of using this technique to track an invasive species. The use of specific primers, especially when combined with next generation sequencing technologies, has allowed the use of eDNA in non-aquatic systems (Valentin et al. 2016). Early detection of invasive species is critical to stopping their spread and proliferation and this work gives another tool for detection of terrestrial invasive arthropods.

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Research Publications:

Macías-Hernández, N., **Athey, K.J.**, Tonzo, V., Wangensteen, O.S., Arnedo, M., Harwood, J.D. 2017. Exploring spider digestive processes in the context of body part DNA extraction and molecular gut content analysis. *Bulletin of Entomological Research* (in review).

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Extension Presentations:

Athey, K.J., Dreyer, J., Williams, M. and Harwood, J.D. **2016**. Alternative cucumber beetle and bacterial wilt management. 2016 Kentucky Fruit and Vegetable Conference, Lexington, KY

Research Presentations:

Invited Oral Presentations

Athey, K.J., Ruberson, J.R., and Harwood, J.D. **2016.** A flood of stink pests: Trophic connections during kudzu bug invasion. NCB-ESA Meeting, Cleveland, OH

Dreyer, J., **Athey, K.J.**, Harwood, J.D., and Williams, M. **2016.** Some lessons learned: Pests and predators in an organic cucurbit system. NCB-ESA Meeting, Cleveland, OH

Offered Oral Presentations

Athey, K.J., Dreyer, J., Williams, M. and Harwood, J.D. **2015**. Biological control and intraguild predation by generalist predators in cucurbits: implications for organic production. ESA Meeting**,** Minneapolis, MN

Sitvarin, M., **Athey K.J.**, Whitney, T.D., and Harwood, J.D. **2015**. Seasonal changes in intraguild predation and prey utilization among wolf spiders: A molecular assessment. ESA Meeting**,** Minneapolis, MN

Penn, H.J., **Athey, K.J.**, and Harwood, J.D. **2015**. Direct and indirect trophic interactions of ants, spiders, and soybean pests. ESA Meeting**,** Minneapolis, MN

Athey, K.J., Dreyer, J., Williams, M. and Harwood, J.D. **2014**. The hidden world: ground predation and its impact on the yield of organic cucurbit crops. ESA Meeting**,** Portland, OR

Athey, K.J. and Harwood, J.D. **2013.** Non-consumptive killing of distasteful pests: Are spiders engaging in superfluous killing? ESA Meeting**,** Austin, TX

Kowles, K.A., **Athey, K.J.**, Johnson, D.W., and Harwood, J.D. **2013.** Tracking aphid predation through molecular and spatial analysis. ESA Meeting, Austin, TX

Athey, K.J. and Harwood, J.D. **2013.** Control of stink bugs by spiders: A possibility or a pipe dream? OVEA Meeting, Indianapolis, IN

Kowles, K.A., **Athey, K.J.**, Johnson, D.W., and Harwood, J.D. **2013.** Sweet, destructive aphids: predation by Coccinellidae in a winter wheat agroecosystems. OVEA Meeting, Indianapolis, IN

Gardiner, M.M., Burkman, C.E. Smith, C.A., Parker, D., **Athey, K.J.,** and Harwood, J.D. **2013.** Can change in urban population size represent an opportunity to enhance predator biodiversity and biocontrol services in shrinking cities? The Ohio State University Department of Entomology Seminar. Wooster, OH

Athey, K.J., Ruberson, J.R., and Harwood, J.D. **2013.** The double-edged sword of biological control: The bean plataspid (*Megacopta cribraria*) and its predators in cotton and soybeans. **NCB-ESA Meeting, Rapid City, SD**

Athey, K.J., Ruberson, J.R., and Harwood, J.D. **2013.** Does prey availability matter for distasteful pests? Investigations of stink bug food webs. MTI-2, Lexington, KY and The University of Kentucky, Center for Ecology, Evolution and Biology Spring Research Symposium (UK-CEEB), Lexington, KY

Kowles, K.A., **Athey, K.J.**, Johnson, D.W., and Harwood, J.D. **2013.** Molecular and spatial analysis of an aphidaphagous predator in winter wheat: Implications for conservation biological control. MTI-2, Lexington, KY and UK-CEEB Spring Symposium, Lexington, KY

Athey, K.J., Ruberson, J.R., and Harwood, J.D. **2012.** The stink bug assassins: Exploring biological control options using molecular techniques. ESA Meeting, Knoxville, TN

Athey, K.J., Ruberson, J.R., and Harwood, J.D. **2012**. The pentatomid hunters: molecular tracking of predation on stink bugs (Hemiptera: Pentatomidae) in cotton and soybeans. **NCB-ESA Meeting, Lincoln, NE and Joint Meeting of the Southeastern and Southwestern Branches of ESA, Little Rock, AK**

Peterson, J.A., **Athey, K.J.**, Chapman, E.G., Harwood, J.D. **2012**. Seasonal patterns in generalist trophic interactions based on molecular gut-content analysis. **NCB-ESA meeting, Lincoln, NE**

Johansen, K.J., Ruberson, J.R., and Harwood, J.D. **2011**. What's eating you? Molecular tracking of predation on stink bugs (Hemiptera: Pentatomidae) in cotton. ESA Meeting, Reno, NV

Peterson, J.A., **Johansen, K.J**., Chapman, E.G., and Harwood, J.D. **2011**. The role of non-prey food resources in generalist predator food webs. ESA Meeting, Reno, NV

Ruberson, J.R., Olson, D.M., **Johansen, K.J**. and Harwood, J.D. **2011**. Natural enemies of stink bugs. ESA Meeting, Reno, NV

J**ohansen, K.J. 2010**. What's that Bug? Creating keys to the subfamilies of Braconidae. Department of Entomology Seminar, University of Kentucky, Lexington, KY

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Johansen, K.J. 2008. Rearing the Beast: Raising caterpillars and parasitoids. Annual Meeting of the Society of Kentucky Lepidopterists, Lexington, KY

Johansen, K.J. 2008. The Caterpillar Hunters: Ecological & phylogenetic studies of host-parasitoid relationships. Department of Entomology Seminar, University of Kentucky, Lexington, KY

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Johansen, K.J. 2006. Dragonflies and damselflies as indicators of restored wetland habitat. University of Nebraska Honors Program Spring Symposium, Omaha, NE

Poster Presentations

Allen, C.D., Haynes, K.F., Obrycki, J.J., **Athey, K.J**., and Harwood, J.D. **2013**. The role of plant chemistry in complex food webs: using GCMS and PCR to reveal predator-prey interactions in an invasive plant system. MTI-2, Lexington, KY

Athey, K.J., Ruberson, J.R., and Harwood, J.D. **2012**. Spiders as Potential Biological Control Agents of Stink Bugs in Cotton and Soybeans. Arachnological Society of America Annual Meeting, Green Bay, WI

Harwood, J.D., Allen, C.D., Chapman, E.G., **Johansen, K.J.**, Kowles, K.A., McKenrick, H.J., Peterson, J.A., Schmidt, J.M., Welch, K.D., Whitney, T.D. 2011. Disentangling the spider's web: insights from complex terrestrial ecosystems. ESA Meeting, Reno, NV

Awards:

- Shelby Stamper Memorial Extension and Outreach Award 2016
- University of Kentucky College of Agriculture, Food and the Environment Outstanding Staff Awards, Technical/Paraprofessional Category 2015 (nominated)
- Second Prize in the Doctor of Philosophy Oral Presentation Competition at Ohio Valley Entomological Association (OVEA) 26th Annual Forum, Indianapolis, IN 2013.

Professional Service:

- UK Dept. of Entomology Departmental Review Committee, student representative 2016
- UK Dept. of Entomology Arthropod Ecologist Faculty Search Committee member 2016
- UK Dept. of Entomology Greenhouse Committee, student representative 2014-current
- UK H. Garman Entomology Club: President 2015-16, Faculty Liaison 2013-15, Treasurer S2008-09
- Meeting Coordinator, Second International Symposium on the Molecular Detection of Trophic Interactions (MTI-2), Lexington, KY May 2013
- NCB-ESA Executive Committee Student Representative 2013-2014
- NCB-ESA Student Affairs Committee: Treasurer 2014-15, Chair 2013-14, Presiding Third Member 2012-13
- UK College of Ag StaffLinks Entomology Department Representative 2011-13
- UK Graduate Council member 2008-09
- Reviewer of 6 manuscripts from Biological Control (3), Molecular Ecology (1), Journal of Pest Science (1), and Food Webs (1)
- Co-organized Specialty Crops Symposium at NCB-ESA 2016
- Co-organized Student Affairs Symposium at NCB-ESA 2012
- Moderator NCB-ESA Symposium 2012, 2016 and OVEA annual meeting 2015
- Student volunteer at national ESA (2008, 2013) and NCB-ESA meetings (2008, 2009, 2012, 2013)

Extension and Outreach:

- STEM Mentoring Panel Participant, Youth Science Summit, June 2016
- Bugs, Bugs, Bugs Earth Day Event, April 2016
- Landsdowne Elementary Arts and Science Day, March 2015
- Science Fair judge, Fayette Co., KY 2014
- Insect Safari at the Arboretum, September 2014
- Lexington Explorium volunteer April, June July, August 2012, July, October 2013
- Raven Run Night Walk July 2009, 2010, 2011, 2012

- "Bugs All Day" at the Lexington Explorium, April 2010, 2012
- "Trees, Trails and Creatures" at the University of Kentucky Arboretum, October 2010, September 2016

Professional Societies:

- Entomological Society of America
- American Arachnological Society
- Gamma Sigma Delta
- Ohio Valley Entomological Society
- Golden Key Honor Society
- Tri-Beta Biological Honor Society

Scholarships/Fellowships:

- North Central Branch of the Entomological Society of America (NCB-ESA) Presidential Student Travel Scholarship 2012 ($250)
- UK Graduate School Travel Grant 2012, 2015 ($400)
- UK Department of Entomology Publication Scholarship, 2010 ($250)
- University of Nebraska Reagents Scholarship, 2002-2006 ($54,000)

Grants:

- UNO College of Arts and Sciences, Thesis Support Grant, 2005 ($700)