SPECIES COMPARISONS OF BOLL FEEDING SUCKING BUGS ON COTTON: ECONOMIC INJURY, DISEASE TRANSMISSION, AND MOVEMENT (HEMIPTERA: PENTATOMIDAE, MIRIDAE)

A Dissertation

by

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ABSTRACT

Injury to cotton, *Gossypium hirsutum* L. (Malvaceae), by a complex boll-feeding sucking pests has increased substantially with the widespread adoption of transgenic Bt (*Bacillus thuringiensis)*-cotton. The resulting insecticide use decline has likely released plant bugs and stink bugs (Hemiptera: Miridae and Pentatomidae, respectively) formerly controlled by broad-spectrum insecticides. Injury from these bugs has been shown to cause decreased fruit retention, boll injury, lint and seed loss. Loss can be further magnified when bacterial boll rot is introduced during probing and feeding activity from insects. Several studies have shown boll injury to cotton by stink bugs and verde plant bug, and management guidance is available for a mixed complex of stink bugs and verde plant bug; however, less is known concerning the comparative boll injury, yield decline, and disease potential for these species and the degree they can be managed collectively or separately.

Therefore, the general goal of this dissertation was to characterize and compare the degree of boll injury, cotton boll rot, and yield depression across multiple insect species and cotton developmental periods to generate species-specific economic injury levels, and explore the impact of at least one environmental factor, water availability, on economic injury levels.

To do this, I first characterized species-specific boll injury and disease impacts on yield. This was achieved by plant caging experiments (Chapter II and III). Secondly, I determined the competency of the boll-feeding sucking pest, *Creontiades signatus* (verde plant bug) and its ability to acquire, transmit, and retain a cotton boll rotting bacteria

(Chapters IV), to complement past studies of stink bugs as cotton boll rot vectors. Finally, I determined the effect of photoperiod on the within-plant distribution of the boll-feeding sucking pest, *Acrosternum hilare* (green stink bug) (Chapter V).

The results showed that boll injury and rot was apparent across a range of boll ages and water stress. Despite this variability, I found that subsequent yield decline attributed to insect feeding was seen primarily under water limiting conditions when plants were infested at mid-bloom. The green stink bugs were primarily distributed in the upper portions of the plant on first position bolls during the day. The results support the use of a common stink bug EIL where multiple species occur and a separate EIL for verde plant bug.

DEDICATION

This dissertation, and all the work contained in it, is dedicated in loving memory to my grandmother Carol Jean Galenbeck and to my family. Particularly my parents Diana (Sam) and the late Carl James Glover, and my aunt Fran Stephens. I owe all my current and future successes to their enduring support and encouragement.

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CHAPTER I

INTRODUCTION

Injury to cotton, *Gossypium hirsutum* L. (Malvaceae), by boll-feeding sucking pests has increased substantially with the widespread adoption of transgenic Bt (*Bacillus thuringiensis)*-cotton cultivars targeting lepidopteran pests (Allen et al. 2008, Luttrell et al. 2015). The resulting insecticide use decline has likely released plant bugs and stink bugs (Hemiptera: Miridae and Pentatomidae, respectively) formerly controlled by broad-spectrum insecticides (Lu et al. 2010). Consistent with higher numbers of these pests, cotton boll injury due to plant bug and stink bug feeding has increased substantially during the last two decades in the southern United States, including Texas (Greene et al. 2001, Hopkins et al. 2009, Luttrell et al. 2015).

A complex of boll-feeding sucking bugs in south Texas is composed of three representative stink bug species and one mirid species. Injury from the green stink bug, *Acrosternum hilare* (Say), and brown stink bug, *Euschistus servus* (Say) (Hemiptera: Pentatomidae), has been shown to cause decreased fruit retention, lint staining, lint loss, and seed loss (Greene et al. 2001). Loss can be further magnified when bacterial boll rot is introduced during probing and feeding activity from stink bugs (Medrano et al. 2015). Soybeans grown along the Texas Gulf Coast harbor the redbanded stink bug, *Piezudorus guildinii* (Westwood) (Hemiptera: Pentatomidae), which causes economic injury in soybean (Vyavhare et al. 2014). Redbanded stink bugs may move into developing cotton as soybean pods begin to senesce and injure cotton similar to other stink bugs (JPG,

personal observation). Historically, the southern green stink bug, green stink bug, and brown stink bug are known to be economic pests of cotton (McPherson et al. 2000).

The verde plant bug, *Creontiades signatus* (Distant) (Hemiptera: Miridae), is a significant cotton pest in south Texas. Armstrong et al. (2013) demonstrated that the verde plant bug readily injured bolls <12-d-old from the first day of bloom (white flower), whereas older bolls incurred little or no injury in a no-choice test. When given a choice of varied-age squares and bolls on a branch, Brewer et al. (2012a) found that older squares and young bolls were preferred, which decreased boll retention and increased subsequent yield decline. Verde plant bug is the predominant mirid species of sucking bugs that occur throughout the Texas Gulf Coast cotton growing region (Brewer et al. 2012b). A related species, *Creontiades distant* (Stal) (Hemiptera: Miridae), is known to injure pre-bloom and early bloom cotton in Australia (Khan et al. 2006).

Verde plant bug is also associated with cotton boll rot (Brewer et al. 2012b) and is a suspected vector of the disease based on similar insect-disease relationships previously documented for stink bugs (Medrano et al. 2007 and 2009). Armstrong et al. (2009) isolated bacteria associated with verde plant bug feeding injury on cotton bolls. Medrano et al. (2016) identified the bacteria *Serratia marcescens* as a boll rot pathogen isolated from bolls fed upon by verde plant bug.

Serratia marcescens is an aerobic, motile bacterium widely recognized (Bull et al. 2010) as an opportunistic, gram-negative bacillus, nosocomial pathogen classified as a member of the division *klebsiella-enterobacter-serratia*, which belongs to family Enterobacteriaceae. *Serratia marcescens* had been associated with cucurbit yellow vine

disease (Lukezic et al. 1982, Zhang et al. 2003) and confirmed pathogenic to a limited number of plant families. More recently *Serratia marcescens* has been shown pathogenic to plants infecting across multiple families spanning cucurbits, sunflower, alfalfa, and peppers (Bruton et al. 2003, Ignatov et al. 2016, Gillis et al 2014).

Serratia marcescens was discovered by Bartolemeo Bizio, a young Italian pharmacist, in 1819 when he identified it as a cause of the bloody discoloration on cornmeal mush. Bizio, demonstrated that the "blood" was caused by a living organism, although at the time believed to be a fungus (Merlino 1924). He named the organism in honor of the Italian physicist, Serratia who invented the steam boat and marcescens, derived from the Latin word "to decay" since Bizio observed that the pigment deteriorated quickly, dissolving from a light-pink material into a purplish-red, viscous form. The ability to form pigment (prodigiosin) is characteristic of *S. marcescens* (Krieg et al.1984). The intensity of which ranges from dark red to pale pink, depending on the age of the colonies and its function still remains largely unknown but is thought to be a byproduct of other processes. More recently Tanaka et al. (2004) showed temperature-dependent prodigiosin production for a select strain. This novel activity was correlated to higher environmental temperatures, *S. marcescens* seems to suppress its own growth and the growth of other bacteria when exposed to elevated temperatures.

Serratia marcescens has a predilection for growth on foodstuffs, especially of the starchy variety, where the pigmented colonies are easily mistaken for drops of blood (Klein, E., 1894, Gaughran et al. 1968). As early as the sixth century B.C., Pythagoras had noted the appearance of a bloody coloration on foodstuffs. *Serratia marcescens* was

originally considered to be an innocuous, nonpathogenic, saprophytic water organism, and it was often used as a biological marker because of its easily recognizable red colonies (Whalen, T.A., 1970, McEntegart et al. 1949, Burket et al. 1937). *Serratia marcescens* is widespread in the environment and the organism has been isolated from water, soil, sewage, foodstuffs and animals (Krieg et al.1984) but it is a rare cause of human disease. However, under experimental conditions, it is pathogenic for mice, rats, guinea pigs, hamsters, turtles and dogs (Hejazi et al. 1997). The *Serratia* species are occasionally recognized as a cause of hospital acquired infections such as urinary tract infections, respiratory tract infections and wound infections (Su et al. 2003, Khanna et al. 2013, Us et al. 2017). In the mid 1800's the organism was referred to as *Monas prodigiosus*, or the "miracle bacterium," which was later modified to *Bacillus prodigiosus.* (Breed et al. 1924). By the 1920's revision in the taxonomy of bacteria as well as the desire to recognize the work of Bizio eventually lead to the adoption of the original name proposed by Bizio, In the bacterial nomenclature, *Serratia marcescens* is now outranked in age only by the genera vibrio (1773) and polyangium (1809) making among the oldest recognized bacteria in the world (Yu 1979).

Currently, management for verde plant bug in south Texas assumes that the boll rot pathogen is present and the insect is capable of transmitting the pathogen. The potential of economic damage is evaluated by checking for signs of internal feeding, including opening green bolls during field monitoring activities (Greene et al. 2001, Brewer et al. 2013) The ability to breach the carpel wall may be associated with mouthpart morpholgy (Esquivel 2016) and possibly other factors. Variablity associated with boll rot disease

transmission is much less studied, limited primarily to the southern green stink bug *Nizara viridula*, (Medrano et al.2007).

Unfortunately, early detection of cotton boll rot infection caused by *S. marcescens* and possibly vectored by verde plant bug is limited since there is an absence of external infection symptoms on immature green bolls, and likely no visible internal symptoms soon after infection as seen by boll rot bacteria vectored by stink bugs (Medrano et al. 2007). Known disease symptoms from cotton boll rotting bacteria introduced by stink bugs include discolored lint and necrotic seed that can be observed only when infected green bolls are cross-sectioned at least two weeks after initial infection or once mature bolls open (Medrano et al. 2007). If transmission potential can be authenticated, then insecticide use decision-making can be revisited and adjusted based on transmission and retention risk.

In general, boll injury to cotton by stink bugs and the verde plant bug has been well documented, and management guidance is available for a mixed complex of stink bugs (Greene et al. 2001) and verde plant bug (Brewer et al. 2013). But less is known concerning the comparative boll injury and yield decline potential for these species and the degree they can be managed collectively or separately. Furthermore, plant water stress may change the sensitivity of cotton to injury by the sucking bug complex as seen for *Lygus* sp. (Hemiptera: Miridae) on cotton (Brewer et al. 2016). This is especially relevant in dryland growing conditions where seasonal rainfall greatly fluctuates, such as is often the case in south Texas (Brewer et al. 2016).

The management of stink bug infestations on cotton currently relies on use of insecticides when action or economic thresholds are exceeding based on visual sampling methods. Methods available include collecting green bolls (2.5 cm in diameter) for internal injury assessment (Toews et al. 2009) and sampling for stink bugs using a drop cloth (Reay-Jones et al. 2009), sweep net (Outward et al. 2008), or beat bucket (Pyke et al. 1980). The sweep net has been found to be more effective at sampling nymphs, while the drop cloth was more effective at sampling adults (Reay-Jones et al. 2009). Conversely, the beat bucket has been found to effective at sampling all life stages, but efficiencies in its use are affected by plant growth stage. Sampling strategies that displace insects for density estimates are further complicated by the remarkable attachment ability of the stink bug with higher attraction forces (>40) greater than its body weight (Voigt et al. 2019). Monitoring plants for both detection and density estimation of stink bugs requires intensive and time-consuming sampling that may be affected by time of day and associated changes in within-plant distribution. Failure to account for such variation may affect the accuracy of detection and population density estimation efforts, with potentially negative management consequences particularly if numbers are underestimated. Biologists have used a wide variety of mark-release techniques to study animal distribution and movement (Southwood 1978). Rice et al. (2015) demonstrated that marking stink bugs with fluorescent powders and using UV LEDs light sources was a simple, nondestructive and effective technique for detecting otherwise difficult to observe aspects of stink bug distribution during nighttime field studies. Cabrera et al. (2016) reported the use of

markers of various colors and found no fitness cost or toxicity associated with the marking agent but noted variability in ability to observe different colors.

Dissertation Research and Relevance

The chapters in this dissertation detail insect derived boll damage, boll rot, and yield loss of boll-feeding stink bugs and plant bugs of South Texas. Three main objectives were entertained: (1) characterize and compare the degree of boll injury, cotton boll rot, and yield depression across multiple insect species and cotton developmental periods to generate species-specific economic injury levels (2) assess the verde plant bug's capacity to introduce boll rot and, (3) assess the photoperiod-specific distribution of the green stink bug within in individual cotton plants.

The research presented in this dissertation aims to compare plant response from a complex of boll-feeding sucking bugs on cotton, and its broadest context to consider if comprehensive management is feasible. All of these species used occur along the Gulf coast region of Texas. Therefore Chapter 2 of this dissertation was to characterize and compare the degree of boll damage, boll rot, and yield depression as a result of feeding activity of three primary stink bugs and a plant bug occurring in Texas, and to calculate economic injury levels based on yield—insect density relationships (Pedigo et al. 1986, Benedict et al. 1989). Based on the results, the extent to which the members of this species complex can be managed individually versus jointly was considered. Caging studies were used to measure whole plant injury and yields (whole plant cages) under plant bug and

stink bug feeding pressure side-by-side to further define the critical period of insect management based on crop developmental stage. Whole plant cages will be used to characterize the effects of multiple insect species at various densities and period of bloom on yield. Further, I investigate the water limiting/ non-water limiting contrast through experimental manipulation of soil moisture by irrigation. The role of plant water-deficit stress on plant response and insect activity by species and density will be used to reevaluate economic injury levels based on yield-insect density relationships.

Chapter 3 investigates and compares fruit retention, boll injury, boll rot, and lint decline as a result of feeding activity on individual bolls varying in age. The same species were considered in this study as used in Chapter 2 in order to examine the extent that boll age sensitivity to feeding and species differences contribute to differences seen in boll injury, boll rot, and yield decline when cotton was infested at mid-bloom and late-bloom on whole plant cage experiments of Chapter 2. Single boll cages were stocked with individual species and were used compare side-by-side across multiple species and multiple boll ages. Here, boll injury can be fully characterized using single boll caging.

In Chapter 4 I investigated verde plant bug disease transmission cycle as a model system to investigate pathogen transmission and retention over time to expand the work that showed an association of verde plant bug with bacterial rot (Chapter 2). The specific objectives of this study were to confirm pathogenicity and transmission of bacteria recovered from bolls fed on by verde plant bug exposed to the bacteria *Serratia marcescens* and examine disease retention as verde plant bug feeds on multiple bolls in a field setting. Verde plant bugs exposed and non-exposed (uninfected) were placed on

individual cotton bolls. These same insects were re-caged on new clean bolls to investigate if the vectors continue to transmit disease. After 2 weeks of boll growth in the field to allow disease development a portion of the bolls were inspected and photographed. Verification of boll rot pathogen introduction by the bugs and measurement of the severity of boll rot were done at the USDA ARS Cotton Pathology Unit in College Station using molecular techniques.

The remainder of the research presented here is aimed at improving the accuracy and precision of insect detection methods. In Chapter 5, I conducted mark-release-observe experiments using a combination of fluorescent marking techniques and blacklight-aided visual observations to determine where (within-plant distribution) stink bugs infesting cotton are observed and whether these patterns are affected by photoperiod when sampling occurs. Field collected stink bugs were individually marked or left unmarked with fluorescent Sharpie markers and were released into interior experimental plots nested within a larger contiguous and uniform cotton fields at peak bloom and monitored to characterize the within-plant vertical distribution of the green stink bug. Stink bugs where monitored visually during day and night, aided by a handheld blacklight for nighttime observations. Within-cotton distribution insect observations were categorized by (i) plant section (i.e. bottom, middle, and top branches), (ii) fruiting positions and leaf surface, and (iii) concealed or exposed position on floral bracts and leaf surfaces. Specifically, I tested whether stink bugs were distributed evenly across plant sections, fruiting positions and leaf surfaces, and concealed or exposed orientation within in those categories, during daytime and nighttime observations.

Together, the objectives of this research will add to the overall understanding of plant response to a boll-feeding sucking bug complex in Texas cotton. Understanding the injury and disease potential for these important pest species has direct relevance to effective pest management. The second chapter documents species-specific susceptibility of cotton to members from the boll-feeding complex using whole plant caging experiments to generate economic injury levels.

The third chapter documents species-specific susceptibility of varied-aged cotton bolls to select members of the boll-feeding complex using single boll caging experiments. Comparisons of fruit retention, boll injury, boll rot, and subsequent yield depression were used to further support experiments from Chapter 4. The fourth chapter used the verde plant bug and a known cotton boll rot pathogen as a model system for exploring disease transmission and retention. The fifth and final chapter explored the photoperiod-specific distribution of the green stink bug within in individual cotton plants. Taken together, these chapters combine elements of applied field ecology, modern vector competency experiments, and contemporary approaches to integrated pest management to provide a well-rounded examination of plant response to a complex of boll-feeding sucking bugs occurring on cotton.

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CHAPTER II

PLANT RESPONSE AND ECONOMIC INJURY LEVELS FOR A BOLL FEEDING SUCKING BUG COMPLEX ON COTTON*

Introduction

Injury to cotton, *Gossypium hirsutum* L. (Malvaceae), by boll-feeding sucking pests has increased substantially with the widespread adoption of transgenic Bt (*Bacillus thuringiensis)*-cotton cultivars targeting lepidopteran pests (Luttrell et al. 2015). The resulting insecticide use decline has likely released plant bugs and stink bugs (Hemiptera: Miridae and Pentatomidae, respectively) formerly controlled by broad-spectrum insecticides (Lu et al. 2010). Consistent with higher numbers of these pests, cotton boll injury due to plant bug and stink bug feeding has increased substantially during the last two decades in the southern United States, including Texas (Greene et al. 2001, Hopkins et al. 2009, Luttrell et al. 2015).

A complex of piercing-sucking boll-feeding insects in south Texas is composed of three representative stink bug species and one mirid species. Injury from the green stink bug, *Acrosternum hilare* (Say), and brown stink bug, *Euschistus servus* (Say) (Hemiptera: Pentatomidae), has been shown to cause decreased fruit retention, lint staining, lint loss, and seed loss (Greene et al. 2001). Loss can be further managed when bacterial boll rot is

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introduced during probing and feeding activity from stink bugs (Medrano et al. 2015). Soybeans grown along the Texas Gulf Coast harbor the redbanded stink bug, *Piezudorus guildinii* (Westwood) (Hemiptera: Pentatomidae), which causes economic injury in soybean (Vyavhare et al. 2014). Redbanded stink bugs may move into developing cotton as soybean pods begin to senesce and injure cotton similar to other stink bugs (JPG, pers. obs.). Historically, the southern green stink bug, green stink bug, and brown stink bug are known to be economic pests of cotton (McPherson et al. 2000).

The verde plant bug, *Creontiades signatus* (Distant) (Hemiptera: Miridae), is a significant cotton pest in south Texas. Armstrong et al. (2013) demonstrated that the verde plant bug readily injured bolls <12-d-old from the first day of bloom (white flower), whereas older bolls incurred little or no injury in a no-choice test. When given a choice of varied-age squares and bolls on a branch, Brewer et al. (2012a) found that older squares and young bolls were preferred, which decreased boll retention and increased subsequent yield decline. Verde plant bug is also associated with cotton boll rot (Brewer et al. 2012b) and is a suspected vector of the disease based on similar insect-disease relationships previously documented for stink bugs (Medrano et al. 2009). Verde plant bug is the predominant mirid species of sucking bugs that occur throughout the Texas Gulf Coast cotton growing region (Brewer et al. 2012b). A related species, *Creontiades distant* (Stal) (Hemiptera: Miridae), is known to injure pre-bloom and early bloom cotton in Australia (Khan et al. 2006).

In general, boll injury to cotton by stink bugs and the verde plant bug has been well documented, and management guidance is available for a mixed complex of stink

bugs (Greene et al. 2001) and verde plant bug (Brewer et al. 2013). But less is known concerning the comparative boll injury and yield decline potential for these species and the degree they can be managed collectively or separately. Furthermore, plant water stress may change the sensitivity of cotton to injury by the sucking bug complex as seen for *Lygus* sp. (Hemiptera: Miridae) on cotton (Parajulee et al. 2018). This is especially relevant in dryland growing conditions where seasonal rainfall greatly fluctuates, such as is often the case in south Texas (Brewer et al. 2016).

The objectives of this study were to compare fruit retention, boll injury, boll rot, and yield as a result of feeding activity of three primary stink bugs and a plant bug occurring in Texas, and to calculate economic injury levels based on yield—insect density relationships (Pedigo et al. 1986, Benedict et al. 1989). Based on the results, the extent to which the members of this species complex can be managed individually versus jointly was considered.

Materials and Methods

Insect Collection and Pre-Infestation Cotton Management

Adult insects used for infesting caged whole cotton plants were collected from several wild and cultivated host plants, including cotton, sorghum, soybean and several seepweeds, *Suaeda* spp. (Chenopodiaceae) (Armstrong 2010). Insects were collected using a modified leaf blower that displaces insects from vegetation and transfers them into an inflatable sock that fits on the opposite end of the blower's fanned nozzle, known as a

KISS-sampler (keep it simple sampler) (Beerwinkle et al. 1997). Verde plant bugs were collected in 2014 and 2015 from a mixture of seepweeds and grain sorghum from milk through hard dough stages (Gerik et al. 2003), and in 2016 on soybean, flowering through full pod development (Bean and Miller 1998). Stink bugs were collected from various pod filling stages of soybean. Insects were captured ≈24 h before infestation on caged cotton plants during each of the three years. All insects were held individually in plastic portion cups for a 24 h fasting period, and inspected to confirm that only healthy adults were used for infesting the caged cotton.

The experiment was conducted in 2014, 2015, and 2016 at the Texas A&M AgriLife Research and Extension Center farm in Corpus Christi, TX. Phytogen 499 WRF (Dow AgroSciences, Indianapolis. IN) cotton seed was planted in early May on 91-m rows and 96-cm row centers at a field site of ≈0.4 ha, resulting in a plant stand of ≈77,800 plants per ha (31,500 plants per acre). In 2014, the region experienced extreme drought (192 mm of rainfall from April 15 to August 1, 23% of the average long-term rainfall for the Texas Coastal Bend region [National Weather Service 2017]), and in 2015 the region experienced abundant rainfall providing ample soil moisture for cotton production (622 mm of rainfall from April 15 to August 1). In 2016, the region experienced moderate drought (324 mm of rainfall from April 15 to August 1). Rainfall estimates were acquired from the Corpus Christi airport weather station located ca. 6.75 km from the experiment (National Weather Service 2017). In 2016, plots were subdivided into water limiting (dryland) and irrigated (non-water limiting) sections to mimic dryland and abundant rainfall conditions, respectively. Cotton plots were grown without irrigation except in

2016 as noted above. Thiamethoxam insecticide (Centric, Syngenta Crop Protection, Greensboro, NC) at labelled rates was used ca. every 10 days to maintain plots pest free before and after infestation. Thiamethoxam application was discontinued 14 days prior to infesting with experimental insects, and application was restarted at the conclusion of the infestation period. Other agronomic practices were normal for the region (Morgan 2018).

Whole Plant Cage Experimental Design and Insect Infestation

Insects were released into caged cotton for a 1-week period to characterize the effects of species and insect density on fruit retention, cotton boll injury, cotton boll rot, and yield. In any year, the experimental design was a species by infestation rate factorial with a minimum of six replications conducted separately at one or two blooming periods and under dryland (water limiting) conditions in 2014, non-water limiting conditions in 2015, and manipulated dryland and irrigated conditions in 2016 (Table 2.1). Mid-bloom was characterized as 10-12 NAWF on the first (mainstem) fruiting position (Kerby et al. 2010), and late-bloom was characterized as 7-9 NAWF. In each year, groups of four whole cotton plants were enclosed by large organza fabric cages (152 by 122 cm, ~240 micron mesh, JoAnn's Fabrics, Hudson, OH) at bloom. Two days before each infestation, plants were sprayed with a short-residual U.V.-sensitive pyrethrin insecticide (0.02% by volume, Bonide products, Oriskany, NY) to remove aphids and other small insects that may contaminate the caged cotton. In 2014, an additional un-caged non-infested control treatment of four plants was established and set out randomly with the rest of the

treatments per the design. Data from this treatment and the caged non-infested treatment in 2014 and 2015 were paired to test for a caging effect.

Table 2.1. Field Experiment Conditions. Key experimental and environmental conditions of the field experiments in 2014-2016.

Year	Bloom Period[a]	Irrigation	Water Availability[b]	Species[c]
2014	Mid	No	192 mm	Pg, Es, Ah
2014	Late	No	192 mm	Pg, Es, Ah
2015	Mid	No	622 mm	Cs, Es, Ah
2015	Late	No	622 mm	Cs, Es, Ah
2016	Mid	No	324 mm	Cs, Es
2016	Mid	Yes	665 mm	Cs, Es

Common infestation rates across all years and experiments; infestation rates of 0 (control), 0.25, 1, and 2 adult bugs per plant; infestation duration was one week.

[a] Cotton phenological stage when infestation occurred. Mid-bloom was characterized as 10-12 NAWF and late-bloom was characterized as 7-9 NAWF.

[b] Rainfall estimates were acquired from the Corpus Christi airport weather station located ca. 6.75 km from the experiment (National Weather Service 2017); in 2016 irrigated plots received a total of 665mm of water during the growing season (341mm from supplemental irrigation).

[c] Species used to infest cotton. Pg = *Piezudorus guildinii* (redbanded stink bug); Es = *Euschistus servus* (brown stink bug); Ah = *Acrosternum hilare* (green stink bug); Cs = *Creontiades distant* (verde plant bug).

The species used and water stress conditions varied by year depending on availability (Table 2.1). In 2014, the brown stink bug, green stink bug, and redbanded stink bug were used for mid-bloom and late-bloom infestations under (water limiting) dryland conditions. In 2015, the verde plant bug, brown stink bug, and green stink bug were used to infest cotton at mid-bloom and late-bloom under abundant rainfall (non-water limiting) conditions. In 2016, the brown stink bug and verde plant bug were used to infest cotton only at mid-bloom in two separate experiments planted side-by-side to simulate both dryland and irrigated conditions. Irrigated plots received a total of 68.5 cm of water during the growing season (458 mm from supplemental irrigation and 227 mm rainfall from April

15 to August 1). Above ground irrigation tape and the clay content of the soil allowed effective soil moisture management in the field.

Cages were infested with adults of each species individually at rates of 0, 0.25, 1, and 2 adult insects per plant (i.e., 0, 1, 4, and 8 bugs per cage, respectively) for 7 days. Infestation rates were based on density estimates of boll-feeding insects associated with boll injury and economic injury in commercial fields (Brewer et al. 2013). The 7-day infestation period was chosen to reflect a commercial field were insects might go undetected during weekly scouting. At the conclusion of the infestation period a sampling of caged treatment plants ($n > 10$) were inspected for active insects, which were observed inside all cages inspected. All caged treatments were then treated with thiamethoxam insecticide on day 7 post-infestation and again on day 14 to eliminate non-target pest damage and remnant treatment insects including nymphs emerging from eggs laid by the adults.

Plant Injury and Yield Measurements

Cages remained in place until harvest, allowing bolls and cotton boll rot introduced by these insects to mature. Hand harvest occurred August 15 (113d-emergence to harvest) in 2014, August 1 (119d-emergence to harvest) in 2015, and August 17 (117d-emergence to harvest) in 2016. At harvest, all of the four individual experimental plants from each cage were mapped using PMAPplus (Anderson et al. 2018) in all three years to record fruit retention, boll injury, and cotton boll rot. Boll injury was rated using a boll injury scale ranging from 0 (representing no locule injury) to 1-3 (representing a progression of

seed and lint degradation occurring in 1-3 locules, respectively) to 4 (representing severe degradation of seed and lint in all locules) (Lei et al. 2003). Mean percent fruit retention per plant was calculated for sympodial branches for each species and infestation rate. All bolls retained on the plant were counted, open bolls were rated, and green bolls were placed in a commercial dryer until fully dried and opened. The boll interior was thoroughly inspected for symptoms of cotton boll rot (Medrano et al. 2009). Bolls were scored on presence or absence of cotton boll rot visually. Yield data were estimated by cotton lint weights. To obtain lint weight, seed cotton was ginned by hand using a 10-saw laboratory cotton gin (Continental Eagle Crop., Prattville, AL). Weights were recorded per plant.

Data Analysis and Economic Injury Level (EIL) Calculation

Percent fruit retention and bolls with symptoms of cotton boll rot were transformed by arcsine square-root transformation of the proportion before analysis because of the wide range of values encountered (Neter et al. 1985). Back transformed means were presented for ease of interpretation. To assess the overall caging effect, yields from the no-cage control were compared with the uninfested caged controls in 2014 and 2015. Analysis of variance (ANOVA) was used, conforming to a randomized complete block of two treatments (uncaged control and uninfested caged control) (Proc GLM, Littell et al. 1991). Based on the lack of differences in 2014 and 2015 and a past study showing no yield differences using the same method (Brewer et al. 2013), caging effects were not further considered (data not presented).

All measurements taken from caged treatments were used to examine treatment effects by using an ANOVA for a two-way factorial (species and infestation rate) (Proc GLM, Littel et al. 1991) including the following specifications. The species and infestation rate factors were set as fixed effects; therefore, the residual was set as the error term for the main effects and the infestation rate-species interaction (Neter et al. 1985). Four infestation rates were used to consider linearity and other higher order polynomial trends across the range of the values. Standard coefficients for linear, quadratic, and cubic orthogonal contrasts (Neter et al. 1985) were applicable because there were few missing data points and no missing treatment combinations. If the interaction was significant, the trend analyses were conducted separately by species. If the interaction was not significant, the same contrasts were used to evaluate trends common across all species (Quinn and Keough 2002). A Tukey's mean separation test ($\alpha = 0.05$) was used to compare results among species averaged across the infestation rates when the species main effect was significant and the interaction was not (Neter et al. 1985).

Field trials conformed to a three (stink bugs species) by four (infestation rate) factorial in 2014 (drought year, mid-bloom and late-bloom infestations), a three (2 stink bugs, 1 plant bug) by four (infestation rates) factorial in 2015 (wet year, mid-bloom and late-bloom infestations), and a two (1 stink bug, 1 plant bug) by four (infestation rates) factorial under dryland and irrigated conditions in 2016 (mid-bloom infestation only) (Table 2.1) (Littell et al. 1991). Measurements were analyzed separately for the two blooming periods in 2014 and 2015 and the two water conditions in 2016 because the experiments were conducted separately.

Analyses using first (simple linear), second (quadratic), and third (cubic) order regression equations (Neter et al. 1985) were used to consider yield response-insect density relationships for significant results of the lint weight measurement. Equations were estimated by species depending on the significance of the infestation rate-species interaction. For linear relationships, $y = mx + b$, were y was the yield response in lint weight per plant, x was the insect density in bugs per plant, b was the y-intercept, and m was the slope in lint weight per bug per plant. Because significant linear relationships across infestation rates were detected, an economic injury level was calculated by using the formula of Pedigo et al. (1986), $EIL = C/(V*I*D*K)$, where C was the expected cost of control, V was the expected market share value of the cotton lint, and K was the expected proportion of the population to be controlled. Variables C, V, and K were set as constants of US$18.53 per ha (US$7.50 per acre), US$1.90 per kg (US$0.86 per lb.), and 0.9 (90% expected control). I was plant injury per insect, and D was yield loss per plant injury unit. Lint weight loss per bug per plant from the experiment was used to estimate the product $I*D$, and was estimated from the slope m, converted to lint weight (hectare basis) per bug by using a conversion factor of 77,800 plants per hectare. The much lower-valued seed weight loss was not included.

Results

Significant differences in yield were not detected between the caged and uncaged treatments in 2014 ($P > 0.22$), confirming minimal to no caging effect on plant growth

and fruiting potential. An ANOVA was run individually for each year and blooming period because species used and growing conditions varied across years (Table 2.1). Significant yield decline was not observed when infestations occurred on late-blooming cotton in 2014 and 2015 (2 ANOVAs, $P > 0.20$) (Figure 2.1); although four of six ANOVAs of the boll measurements had significant factors. In contrast, yield differences were observed exclusively during the mid-bloom period (4 ANOVAs, $P < 0.03$) (Figure 2.2) and twelve of the fruit retention, boll injury, and boll rot measurement ANOVAs for the mid-bloom period of infestation had significant factors. All analysis below will focus on the mid-bloom period of infestation, where the significant boll measurements were followed by significant yield differences across treatments.

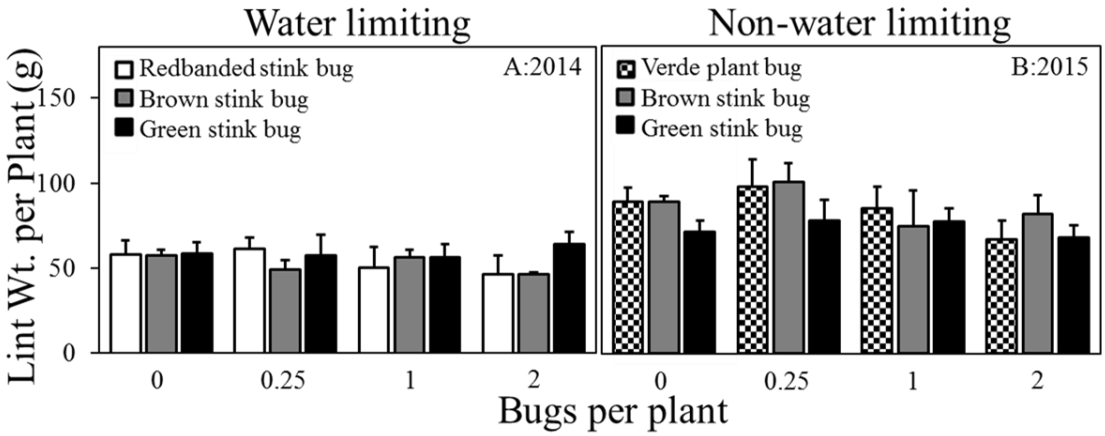

Figure 2.1. Mean Lint Weights for Late-bloom Infestation. Mean (±SEM) lint weights for late-bloom period of infestation under water limiting conditions (A:2014) and non-water limiting conditions (B:2015). Infestation rates of 0 (control), 0.25, 1, and 2 insects per plant; infestation duration was one week. See legend for species used each year.

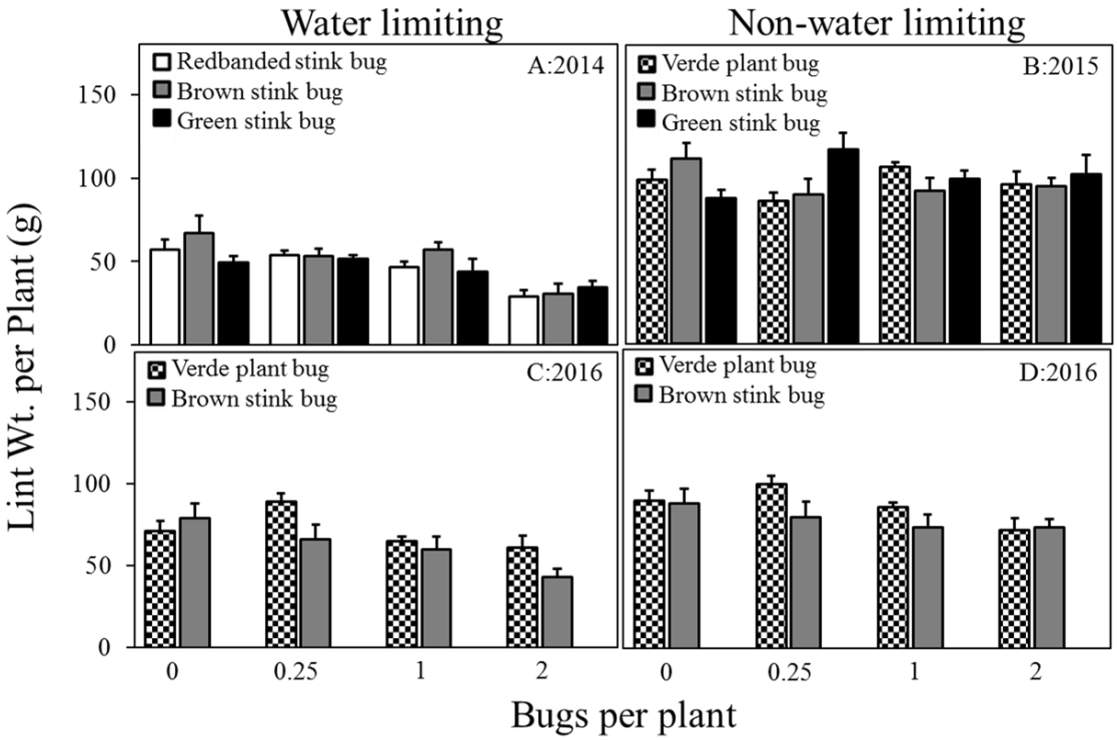

Figure 2.2. Mean Lint Weights for Mid-bloom Infestation. Mean (±SEM) lint weights for mid-bloom period infestation under water limiting conditions (A:2014 and C:2016) and non-water limiting conditions (B:2015 and D:2016). Infestation rates of 0 (control), 0.25, 1, and 2 insects per plant; infestation duration was one week. See legend for species used each year.

Fruit Retention

In 2014, a significant stink bug infestation rate-species interaction was detected when cotton was infested with all three stink bug species at mid-bloom (Table 2.2) (Figure 2.3A). In 2015, a significant interaction was not detected at mid-bloom when cotton was infested with the verde plant bug, brown stink bug, and the green stink bug ($P > 0.05$) (Table 2.2) (Figure 2.3B), or in 2016 when cotton was infested with the verde plant bug and brown stink bug ($P > 0.05$) (Table 2.2) (Figures 2.3C and 2.3D). In 2014, when the interaction was seen trend analyses were conducted separately by species. Fruit retention decreased in a linear fashion as infestation rate increased for the brown stink bug (linear

contrast F = 5.29; df = 1, 16; P = 0.035), but a downward linear trend was not significant for the redbanded or green stink bug (P > 0.05) (Figure 2.3A). In 2015, a significant linear contrast was not detected across the infestation rates for the three species (P > 0.05) (Figure 2.3B). In 2016 (dryland experiment), a downward linear trend as infestation rate increased was significant for brown stink bug (linear contrast F = 16.49; df = 1, 40; P = 0.0002) but not verde plant bug (P > 0.05 (Figure 2.3C). In 2014, the species main effects was statistically significant when the interaction was not significant (P > 0.05, see above). A Tukey test (α = 0.05) indicated that the verde plant bug infestation resulted in significantly lower fruit retention compared to fruit retention for the brown stink bug and green stink bug infestations (Figure 2.3B).

Table 2.2. ANOVA Significance Results for Whole Plant Cages. ANOVA significance tests of the factors, infestation rate, species, and their interaction, conducted separately during mid-bloom and late-bloom (2014, 2015), and during mid-bloom in water limiting (Dryland) and non-water limiting (Irrigated) conditions (2016).

	2014		2015		2016 (Mid-bloom)	
Factor [a]	Mid-bloom	Late-bloom	Mid-bloom	Late-bloom	Dryland	Irrigated
			Yield			
Infestation rate	F = 13.63 df = 3, 58 **P = 0.0001**	F = 3.31 df = 3, 61 **P = 0.026**	F = 0.05 df = 3, 47 P = 0.98	F = 1.53 df = 3, 48 P = 0.21	F = 2.05 df = 3, 40 P = 0.12	F = 3.31 df = 3, 40 **P = 0.029**
Species	F = 2.02 df = 2, 58 P = 0.14	F = 0.12 df = 2, 61 P = 0.88	F = 0.50 df = 2, 47 P = 0.60	F = 1.44 df = 2, 48 P = 0.24	F = 13.62 df = 1, 40 **P = 0.0007**	F = 6.2 df = 1, 40 **P = 0.017**
Infestation rate * Species	F = 1.02 df = 6, 58 P = 0.42	F = 0.95 df = 6, 61 P = 0.46	F = 2.96 df = 6, 47 **P = 0.015**	F = 0.41 df = 6, 48 P = 0.87	F = 3.71 df = 3, 40 **P = 0.019**	F = 2.06 df = 3, 40 P = 0.12
			Fruit Retention			
Infestation rate	F = 0.88 df = 3, 60 P = 0.46	F = 1.01 df = 3, 60 P = 0.39	F = 0.63 df = 3, 47 P = 0.60	F = 1.85 df = 3, 48 P = 0.15	F = 5.72 df = 3, 40 **P = 0.0024**	F = 1.98 df = 3, 40 P = 0.13
Species	F = 4.20 df = 2, 60 **P = 0.019**	F = 1.16 df = 2, 60 P = 0.31	F = 4.80 df = 2, 47 **P = 0.012**	F = 1.42 df = 2, 48 P = 0.31	F = 1.86 df = 1, 40 P = 0.17	F = 1.37 df = 1, 40 P = 0.24
Infestation rate * Species	F = 2.45 df = 6,60 **P = 0.034**	F = 0.94 df = 6, 60 P = 0.47	F = 1.98 df = 6, 47 P = 0.087	F = 0.90 df = 6, 48 P = 0.50	F = 1.61 df = 3, 40 P = 0.20	F = 0.96 df = 3, 40 P = 0.42

Table 2.2. Continued.

	2014		2015		2016 (Mid-bloom)	
Factor [a]	Mid-bloom	Late-bloom	Mid-bloom	Late-bloom	Dryland	Irrigated
			Boll Injury			
Infestation rate	*F* = 46.50 df = 3, 60 *P* = 0.41	*F* = 55.01 df = 3, 59 ***P* = 0.0001**	*F* = 23.49 df = 3, 47 ***P* =0.0001**	*F* = 41.72 df = 3, 48 ***P* = 0.0001**	*F* = 13.26 df = 3, 40 ***P* = 0.0001**	*F* = 4.13 df = 3, 40 *P* = 0.45
Species	*F* = 3.21 df = 2, 60 ***P* = 0.047**	*F* = 2.36 df = 2, 60 *P* = 0.10	*F* = 9.98 df = 2, 47 ***P*= 0.0002**	*F* = 13.92 df = 2, 48 ***P* = 0.0001**	*F* = 30.98 df = 1, 40 ***P* = 0.0001**	*F* = 3.71 df = 1, 40 *P* = 0.18
Infestation rate * Species	*F* = 0.84 df = 6, 60 *P* = 0.54	*F* = 1.08 df = 6, 60 *P* = 0.38	*F* = 2.46 df = 6, 47 ***P* = 0.037**	*F* = 3.96 df = 6, 48 ***P* = 0.0027**	*F* = 9.92 df = 3, 40 ***P* = 0.0001**	*F* = 6.72 df = 3, 40 *P* = 0.45
			Boll Rot			
Infestation rate	*F* =29.48 df = 3, 60 ***P* = 0.0001**	*F* = 41.5 df = 3, 60 ***P* = 0.0001**	*F* = 24.57 df = 3, 47 ***P* = 0.0001**	*F* = 47.35 df = 3, 48 ***P* = 0.0001**	*F* = 12.35 df = 3, 40 ***P* = 0.0001**	*F* = 39.43 df = 3, 40 ***P* = 0.0001**
Species	*F* = 1.55 df = 2, 60 *P* = 0.22	*F* = 2.03 df = 2, 60 *P* = 0.13	*F* = 7.86 df = 2, 47 ***P* = 0.019**	*F* = 13.49 df = 2, 48 ***P* = 0.0001**	*F* = 26.25 df = 1, 40 ***P* = 0.0001**	*F* = 100.77 df = 1, 40 ***P* = 0.0001**
Infestation rate * Species	*F* = 0.32 df = 6, 60 *P* = 0.92	*F* = 0.46 df = 6, 60 *P* = 0.88	*F* = 1.09 df = 6, 47 *P* = 0.38	*F* = 3.87 df = 6, 48 ***P* = 0.0031**	*F* = 4.10 df = 3, 40 ***P* = 0.012**	*F* = 17.48 df = 3, 40 ***P* = 0.0001**

Exact probabilities (*P*) are given, bolding is used to focus attention on significant results of the interaction, P < 0.05, or significant main effects when the interaction is not significant. The residual was used as the error term.

[a] Infestation rate = 0, 0.25, 1, and 2 insects per plant; Species are redbanded stink bug, brown stink bug, and green stink bug in 2014, verde plant bug, brown stink bug, and green stink bug in 2015, and verde plant bug and brown stink bug in 2016.

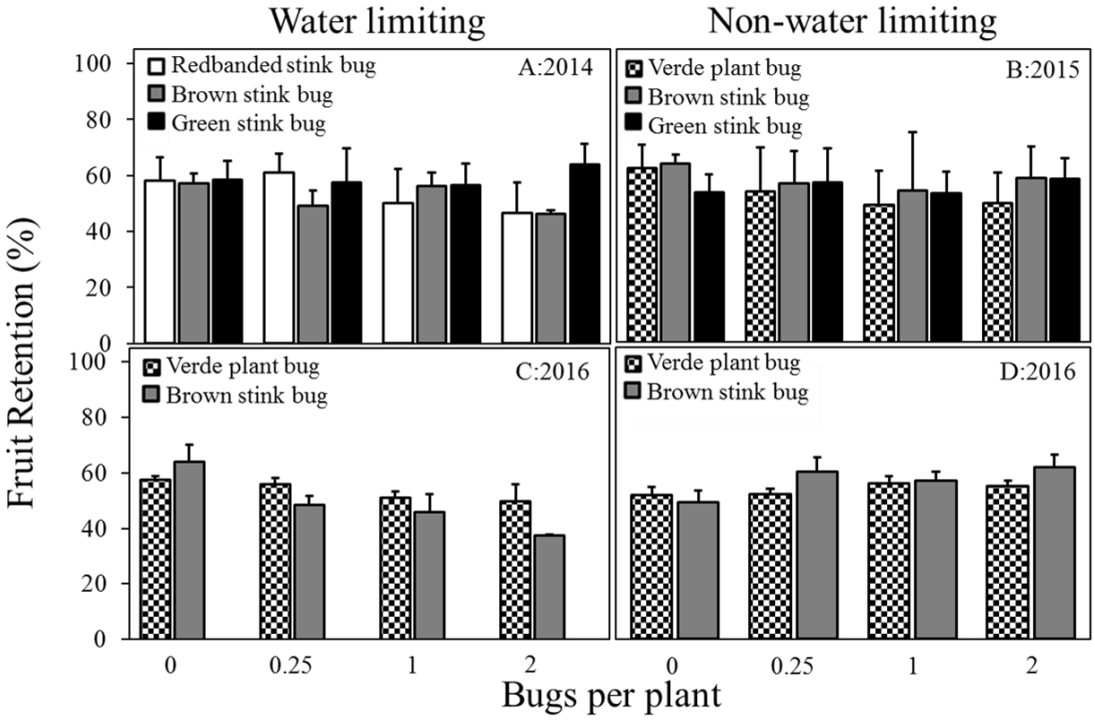

Figure 2.3. Mean Percent Fruit Retention for Mid-bloom Period of Infestation. Mean (±SEM) percent fruit retention for mid-bloom period infestation under water limiting conditions (A:2014 and C:2016) and non-water limiting conditions (B:2015 and D:2016). Infestation rates of 0 (control), 0.25, 1, and 2 insects per plant; infestation duration was one week. See legend for species used each year.

Boll Injury

In 2014, decomposing the models by the interaction, and main effects only when the interaction was not significant, a significant stink bug infestation rate-species interaction was not detected when infested with the redbanded, brown stink bug and the green stink bug at mid-bloom. In addition, no significant differences were detected in 2016 when infested with the verde plant bug and brown stink bug for the irrigated plots ($P >$ 0.05) (Table 2.2) (Figures 2.4A and 2.4D). In 2015, significant stink bug and plant bug infestation rate-species interactions were detected at mid-bloom when infested with the verde plant bug, brown stink bug, and the green stink bug (Table 2.1) (Figure 2.4B). In

2016, significant differences were also detected in dryland conditions when infested with the verde plant bug and brown stink bug (Table 2.1) (Figure 2.4C).

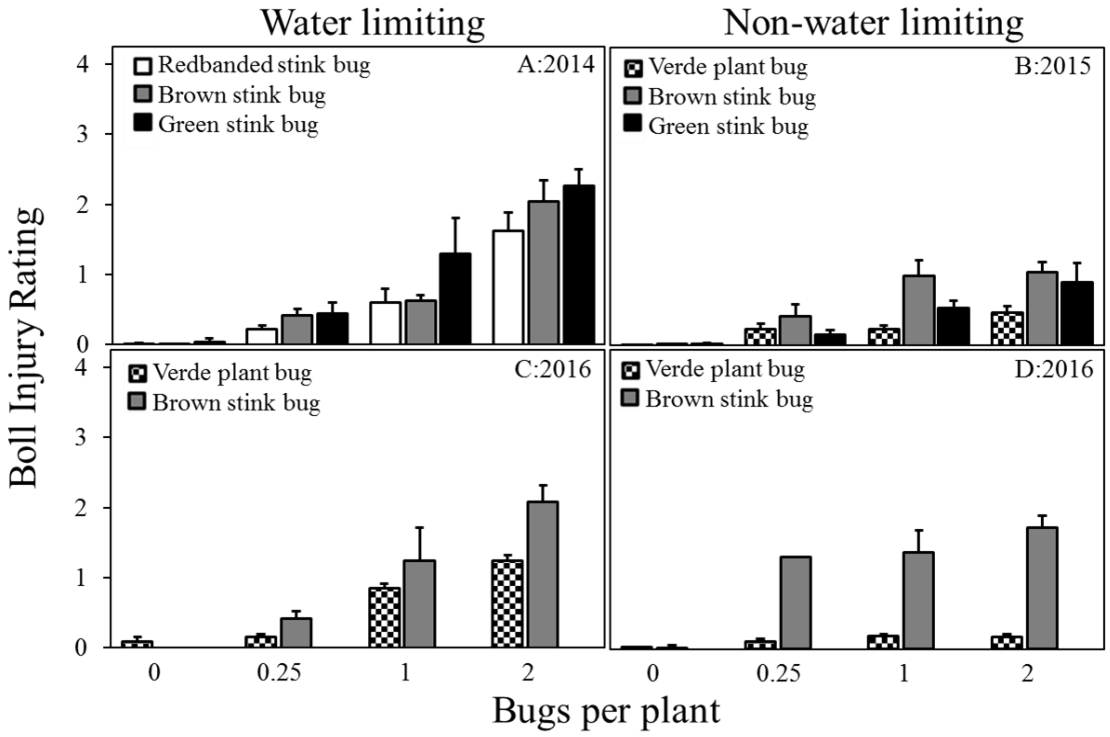

Figure 2.4. Mean Boll Injury for Mid-bloom Period of Infestation. Mean (±SEM) boll injury rating (0-4) for mid-bloom infestation under water limiting conditions (A:2014 and C:2016) and non-water limiting conditions (B:2015 and D:2016). Infestation rates of 0 (control), 0.25, 1, and 2 insects per plant; infestation duration was one week. See legend for species used each year.

In 2014 looking at the main effects, boll injury increased in a linear fashion as infestation rate increased for mid-bloom infestations (linear contrast F = 128.59; df = 1, 71; P = 0.0001) (Figure 2.4A). In 2015, trend analyses were conducted separately by species (the interaction was significant), boll injury increased in an upward linear fashion as infestation rate increased at mid-bloom for the verde plant bug under water limiting

conditions (linear contrast F = 22.74; df = 1, 16; P = 0.0002), brown stink bug (linear contrast F = 28.22; df = 1, 16; P = 0.0001), and the green stink bug (linear contrast F = 22.74; df = 1, 16; P = 0.0002) (Figure 2.4B). In 2016 for the dryland experiment, boll injury was marginally non-significant, tending to increase in an upward linear fashion as infestation rate increased for the verde plant bug (linear contrast F = 4.26; df = 1, 20; P = 0.0523), and brown stink bug (linear contrast F = 35.47; df = 1, 20; P = 0.0001) (Figure 2.4C).

Overall, the brown stink bug and verde pant bug infested plants consistently experienced increased boll injury when compared to uninfested plants. The mirid species comparator, verde plant bug, was less damaging to bolls with most boll injury occurring under dryland conditions at mid-bloom for this species. The verde plant bug was observed to injure cotton in a linear fashion similar to stink bugs but was only economically significant when infestation occurred on mid-bloom cotton under dryland conditions (Figure 2.4C). These results were consistent with previous reports of verde plant bug injury to younger economically significant bolls (Brewer et al. 2013).

Cotton Boll Rot

When visually inspecting open bolls at harvest, up to 29% of bolls had symptoms of cotton boll rot in 2014, 22% in 2015, and 45% in 2016 (Figure 2.5). These results were consistent with previous findings of occurrence and magnitude of cotton boll rot symptoms (Medrano et al. 2015, Brewer at al. 2012b). In contrast to boll injury, an infestation rate-species interaction was detected in 2015 late-bloom, and in 2016 dryland

and irrigated plots during mid-bloom (Table 2.2). In 2016, cotton boll rot progressively worsened as the verde plant bug infestation rate increased (dryland: linear contrast F = 30.60; df = 1, 20; P = 0.0001, Figure 2.5C; irrigated: linear contrast F = 96.76; df = 1, 20; P = 0.0001, Figure 2.5D). When cotton was infested with brown stink bug, the rate of cotton boll rot increase was much steeper (dryland linear contrast F = 6.36; df = 1, 20; P = 0.0202, Figure 2.5C; irrigated linear contrast F = 14.99; df = 1, 20; P = 0.0010, Figure 2.5D). In 2015, the interaction was observed during late-bloom infestations under ample rainfall, possibly reflecting an environment more conducive to boll rot transmission by at least some of the species.

In 2014 and 2015 when an interaction was not detected at mid-bloom, cotton boll rot incidence averaged across species increased in a linear fashion as infestation rate increased in both years (2014: linear contrast F = 86.09; df = 1, 60; P = 0.0001, Figure 2.5A; 2015: linear contrast F = 68.23; df = 1, 47; P = 0.0001, Figure 2.5B). In 2014, significant species main effects (interaction was not significant) on cotton boll rot were not detected (P > 0.05), supporting that the three stink bug species elicited a similar plant response in terms of cotton boll rot under water limiting growing conditions. In 2015, a significant species main effect was detected (Table 2.2), and a Tukey test (α = 0.05) indicated that the brown stink bug induced significantly higher percentage of cotton boll rot compared to that for the verde plant bug and the green stink bug at mid-bloom (Figure 2.5B).

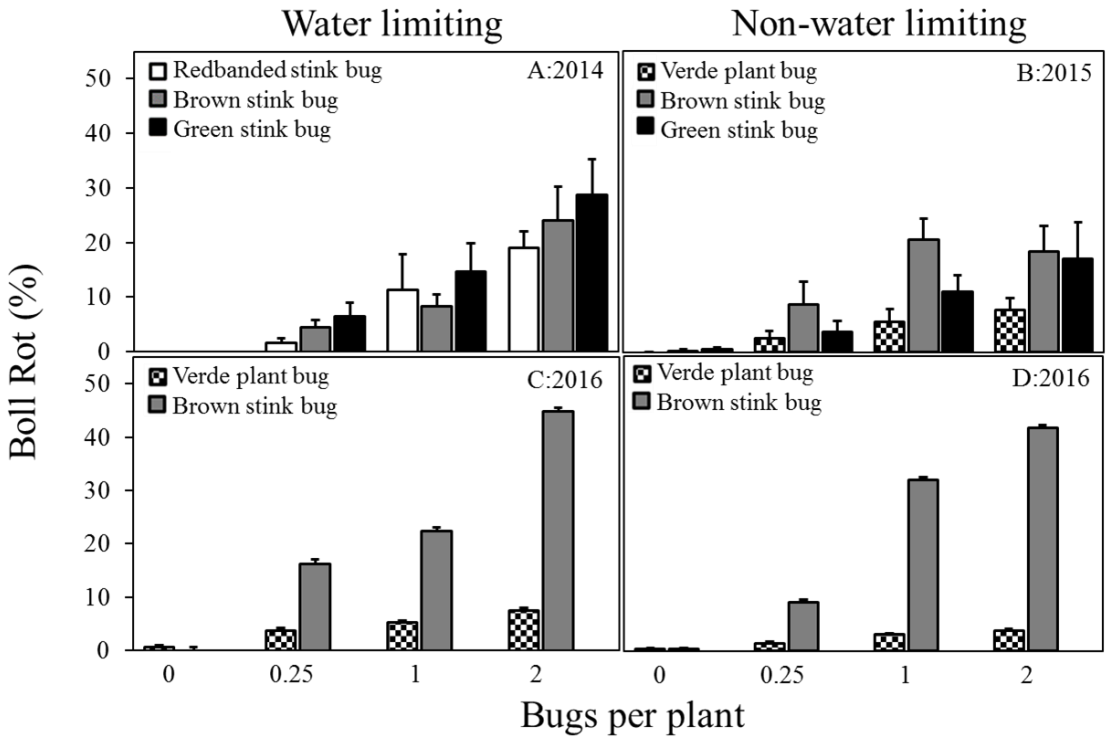

Figure 2.5. Mean Percent Boll Rot for Mid-bloom Period of Infestation. Mean (±SEM) percent boll rot for mid-bloom period infestation under water limiting conditions (A:2014 and C:2016) and non-water limiting conditions (B:2015 and D:2016). Infestation rates of 0 (control), 0.25, 1, and 2 insects per plant; infestation duration was one week. See legend for species used each year.

In general, boll rot seemed to track boll injury; as boll injury increased so did boll rot. Along with the boll injury, these data support mid-bloom as the significantly more sensitive blooming period susceptible to sucking bugs. Verde plant bug inflicted cotton boll injury at about the same level as stink bugs but caused similar or higher fruit abscission losses and less cotton boll rot. The low percentage of cotton boll rot (<10%) observed in the verde plant bug infested plants may be partly attributed to significantly lower fruit retention (Figures 2.3B and 2.3C). In 2016, the significant stink bug and plant bug infestation rate-species interactions detected at both irrigated and dryland production conditions suggest both species are significant contributors to cotton boll rot and provide

further supporting evidence that the brown stink bug is likely a more serious vector of boll rotting pathogens compared to the other stink bug species evaluated.

Yield Response

In 2014, an infestation rate-species interaction was not detected when infested with the redbanded, brown stink bug, and green stink bug at either phenological stages of cotton (Table 2.2) (Figures 2.1A and 2.2A). In 2015, a significant infestation rate-species interaction was detected when infested with the verde plant bug, brown stink bug, and the green stink bug during mid-bloom when rainfall was ample (Table 2.2) (Figure 2.2B), and also in 2016 water limiting (dryland) experiments when infesting mid-bloom cotton with verde plant bug and brown stink bug (Table 2.2) (Figure 2.2C). Although the interaction was not significant in the 2016 irrigated plots, both species and infestation rate main effect were significant (Table 2.2). In 2015, trend analyses were conducted separately by species (the interaction was significant). A significant curvilinear trend was detected at mid-bloom for the verde plant bug (cubic contrast $F = 6.45$; df = 1, 19; $P = 0.021$) (Figure 2.2B) and no linear or curvilinear relationships were detected for the brown stink bug or green stink bug ($P > 0.05$) under ample rainfall conditions. In 2016, a significant downward linear trend was detected for yield as infestation rate increased for infestations occurring at mid-bloom under water-limiting (dryland) condition for the brown stink bug (linear contrast $F = 12.20$; df = 1, 20; $P = 0.0023$) (Figure 2.2C), and no linear or curvilinear relationships were detected for the verde plant bug ($P > 0.05$) (Figure 2.2C). In 2014 when no interaction was detected, yield declined in a downward linear fashion as infestations increased from

0 to 2 bugs per plant for mid-bloom infestations (mid-bloom linear contrast $F = 35.23$; df = 1, 58; $P = 0.0001$, Figure 2.2A). In 2016 under irrigated conditions and when no interaction was detected, yield was lower under brown stink bug infestation than under verde plant bug infestation (Table 2.2) (Figure 2.2D).

The linear decline in fruit retention and increased boll injury as infestation rates increased (Figures 2.3 and 2.4) generally support the interpretation that one of the main causes of yield decline was poor fruit retention and boll injury when plants were infested during mid-bloom under water limiting conditions, but differences across species may be occurring to some degree as reflected in the significant interaction. Cotton boll rot may further magnify the problem during mid-bloom infestation and possibly during late-bloom (Figure 2.5). Declines in fruit retention and yield loss were not detected in plants infested at late-bloom (Figure 2.1), even though both cotton boll injury and cotton boll rot increased as infestation rates increased at late-bloom.

Economic Injury Level (EIL) and Integrated Pest Management Decision Making

A simple linear response best described the yield response-insect density relationship observed for the boll-feeding insects when the ANOVA model was significant, and few higher order polynomial trends were significant. This result supported calculation of EILs using Pedigo's et al. (1986) method, which assumes linearity of the yield response-insect density relationship. Calculation of EILs was most appropriate for data sets of mid-bloom infestation under water limiting conditions in 2014 and 2016. In these data sets, either the infestation rate by species interaction or the main effects for the

yield measurement were significant and followed the significant effects seen in the other measurements. In 2014, the interaction was not significant, and the infestation rate main effect was significant. This justified calculation of a common EIL for the three stink bug species used in this experiment (Table 2.3). For instances where one species dominated, species-specific EILs may be more appropriate; therefore, these values were also calculated (Table 2.3). In 2016 under water limiting conditions, the species by infestation rate interaction was significant; therefore, separate EILs were calculated for verde plant bug and brown stink bug used in this experiment (Table 2.3).

Table 2.3. Economic Injury Levels. Economic injury level (EIL) calculations for three species of stink bugs and verde plant bug when infestation occurred at mid-bloom under water limiting conditions in 2014 and 2016.

Species	Linear regression[a] ($Y=mx+b$)	P [a]	R^2 [a]	I*D[b]	EIL[c] Bugs/plant	EIL[c] Bugs/m--row
	2014					
Redbanded stink bug	Y = -0.42x+34.72	<0.0001	0.59	32.69	0.33	1.32
Brown stink bug	Y = -0.45x+19.71	<0.0001	0.35	35.01	0.31	1.24
Green stink bug	Y = -0.28x+29.54	<0.0001	0.24	21.78	0.50	2.00
Common	Y = -0.41x+51.28	0.0013	0.38	31.89	0.34	1.36
	2016					
Verde plant bug	Y = -0.29x+28.8	<0.0001	0.49	22.14	0.49	1.96
Brown stink bug	Y = -0.47x+29.6	<0.0001	0.36	37.02	0.29	1.16

Common economic thresholds (labeled common in table) across species were calculated only when the infestation rate by species interaction was significant in 2014. Individual species calculations were retained in 2016 when the interaction was significant, therefore individual species EILs were given.

[a] Y was the yield response in lint weight (kg), m was the slope in lint weight per bug per plant, x was insect density in bugs per plant, and b was the y-intercept. P and R^2 indicate probability and fit of the data of the regression line.

[b] I was plant injury per insect, and D was yield loss per plant injury unit. Lint weight loss per bug per plant from the experiment was used to estimate the product I*D, and was estimated from the slope m, converted to lint weight (hectare basis) per bug by using a conversion factor of 77,800 plants per hectare.

[c] EIL = C/ (V*I*D*K), where C was the expected cost of control set at US$18.53 per ha, V was the expected market share value of the cotton lint set at US$1.90 per kg, and K was the expected proportion of the population to be controlled set at 90%.

Discussion

This study illustrates the importance of a complex of sucking bugs as economic pests of cotton, and highlights how variation of at least one environmental factor, water availability, can affect economic injury levels. Our cage study spanned three years and was conducted under both water limiting and non-water limiting conditions that are representative of the region. Based on significant measures during the mid-bloom period of infestation in 2014 and 2015, infestation in 2016 was only done at mid-bloom to further define cotton's sensitivity to multiple species of sucking bugs and variable infestation rates on dryland and irrigated cotton. Cotton vulnerability to and injury potential of boll-feeding sucking bugs are largely determined by fruit age (Allen et al. 2009). The mid-bloom period of cotton development contains the largest array of susceptible boll ages and represents the more sensitive growth period. Significant yield loss during mid-bloom was consistent with previous findings indicating that verde plant bug feed on large squares and small bolls which can result in higher boll abscission under dryland growing conditions (Brewer et al. 2012a). Significant yield losses were associated with verde plant bug infestations in cotton when they occurred at mid-bloom and losses were higher under water limiting conditions (dryland) (Figures 2.2A and 2.2C) as previously seen for cotton fleahopper (Brewer et al. 2016) and *Lygus hesperus* (Parajulee et al. 2018). Similarly, stink bugs have been shown to feed primarily on smaller bolls that occur throughout mid-bloom and to a lesser degree after peak-bloom (Greene et al. 2001). Along with the information provided by others (Greene et al. 2001, Armstrong et al. 2013), the results here were most applicable

early through mid-bloom under water limiting conditions that may be experienced under dryland cotton production.

Khan et al. (2006) showed that the green mirid, *Creontiades dilutus* (Stal) (Hemiptera: Miridae), significantly depressed cotton yield in early blooming cotton in Australia and reported a nominal threshold of 1 to 3 bugs per m-row. By using the 77,800 plants per hectare conversion factor and 96-cm row spacing, our EIL of 0.49 verde plant bug per plant converts to 1.96 verde plant bugs per m-row (Table 2.3), which is within the range provided by Khan et al. (2006). These results were also consistent with an EIL of 0.45 bugs per plant, previously calculated for verde plant bug, but not compared side by side with other species (Brewer et al. 2013).

The EILs for brown stink bug in the two years were similar (0.31 and 0.29 for 2014 and 2016, respectively) and were also similar to that for redbanded stink bug in 2014 (0.33) (Table 2.3), further justifying a common stink bug EIL for at least these two species. The EIL for green stink bug in 2014 was numerically higher (0.50) but did not significantly differ from the other stink bug species. Further experimentation for green stink bug may be warranted, but currently it seems prudent to use the lower common stink bug EIL of 0.34, particularly where these species occur as a complex in the southeast U.S. (Greene et al. 2001) and the Upper Gulf Coast of Texas and central Texas (Brewer et al. 2012b, Suh et al. 2013). Using the conversion of bugs per plant to bugs per m-row (Table 2.3), economic injury levels ranged from 1.16 to 1.24 bugs per m-row for the brown stink bug, 1.32 bugs per m-row for the redbanded stink bug, and 2.0 bugs per m-row for the green stink bug. The converted stink bug EILs were consistent with Greene et al. (2001) who

reported treatment at one bug per 2-m of row (0.5 bugs per m-row) for the green stink bug, southern green stink bug, and brown stink bug provided adequate yield protection. Greene et al. (2001) used the threshold concept, which is appropriately lower than the EIL we report here. More recently, Soria et al. (2017) demonstrated that for a related species, the neotropical brown stink bug, *Euschistus heros* (F.), an economic injury level of 0.5 adult bugs per plant or 2 bugs per m-row preserved yield. Our brown stink bug EIL estimate of 0.31 bugs per plant was lower, but it was calculated in water limiting conditions where cotton is likely more sensitive to insect injury.

Overall, the narrowness in the range of economic injury levels for the complex of stink bugs supports the construction and use of a common stink bug EIL of 0.34 bugs per plant. Verde plant bug was less damaging on average and can be considered separately from the stink bugs using a higher EIL of 0.49 bugs per plant (Table 2.3). If a particular stink bug species dominates in an area, the species-specific EIL for a stink bug also may be more appropriate. In a mixed species situation, the common stink bug threshold of 0.34 bugs per plant is a reasonable approach for pest management.

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CHAPTER III

COMPARISONS OF BOLL INJURY CAUSED BY FIVE SPECIES OF BOLL-FEEDING SUCKING INSECTS

Introduction

Injury to cotton, *Gossypium hirsutum* L. (Malvaceae), by boll-feeding sucking pests has increased substantially with the widespread adoption of transgenic Bt (*Bacillus thuringiensis)* cotton cultivars targeting lepidopteran pests (Allen et al. 2009, Luttrell et al. 2015, Glover et al. 2019). The resulting insecticide use decline has likely released plant bugs and stink bugs (Hemiptera: Miridae and Pentatomidae, respectively) formerly controlled by broad-spectrum insecticides (Lu et al. 2010). As a result, cotton boll injury due to plant bug and stink bug feeding has increased substantially during the last two decades in the southern United States (Luttrell et al. 2015) and elsewhere (Khan et al. 2006, Lu et al. 2010, Soria et al. 2017).

A complex of piercing-sucking boll-feeding insects in south Texas is composed of three representative stink bug species and one mirid species. These species or related species also occur in other locations in the southern United States (Greene et al. 2001, Suh et al. 2013). Injury from the green stink bug, *Acrosternum hilare* (Say), and brown stink bug, *Euschistus servus* (Say) (Hemiptera: Pentatomidae), has been shown to cause decreased fruit retention, lint staining, lint loss, and seed loss (Greene et al. 2001). Loss can be further magnified when bacterial boll rot is introduced during probing and feeding

activity from stink bugs (Medrano et al. 2015). Soybeans grown along the Texas Gulf Coast harbor the redbanded stink bug, *Piezudorus guildinii* (Westwood) (Hemiptera: Pentatomidae) (Vyavhare et al. 2014), which may move into developing cotton as soybean pods begin to senesce (JPG, pers. obs.). Historically, the southern green stink bug, green stink bug, and brown stink bug are known to be economic pests of cotton (McPherson et al. 2000).

The verde plant bug, *Creontiades signatus* (Distant) (Hemiptera: Miridae), is a significant cotton pest in south Texas. Armstrong et al. (2013) demonstrated that the verde plant bug readily injured bolls <12-d-old from the first day of bloom (white flower), whereas older bolls incurred little or no injury in a no-choice test. When given a choice of varied-age squares and bolls on a branch, Brewer et al. (2012a) found that older squares and young bolls were preferred, which decreased boll retention and increased subsequent yield decline. Verde plant bug was also associated with cotton boll rot (Brewer et al. 2012b) and is a suspected vector of the disease based on similar insect-disease relationships previously documented for stink bugs (Medrano et al. 2009). A related species, *Creontiades distant* (Stal) (Hemiptera: Miridae), has been shown to injure pre-bloom and early-bloom cotton in Australia (Khan et al. 2006).

Glover et al. (2019) compared several species of stink bugs and the verde plant bug to generate economic injury levels using whole plant caging experiments. They found severity of boll injury and yield decline was greater when cotton was infested mid-bloom compared to late-bloom. Cross species difference occurred but were less apparent. The objectives of this study were to compare fruit retention, boll injury, boll rot, and lint

decline as a result of feeding activity on individual bolls varying in age. The same species were considered in this study as used in Glover et al. (2019) in order to examine the extent that boll age sensitivity to feeding and species differences contribute to differences seen in boll injury, yield decline, and result in previously observed differences in specific damage and yield when cotton is infested mid-bloom and late-bloom.

Materials and Methods

Insect Collection and Pre-Infestation Cotton Management

Adult insects used for infesting caged single cotton bolls were collected from several wild and cultivated host plants, including cotton, sorghum, soybean and several seepweeds, *Suaeda* spp. (Chenopodiaceae) (Armstrong 2010). Insects were collected using a modified leaf blower that displaces insects from vegetation and transfers them into an inflatable sock that fits on the opposite end of the blower's fanned nozzle, known as a KISS-sampler (keep it simple sampler) (Beerwinkle et al. 1997). Verde plant bugs were collected from a mixture of seepweeds and grain sorghum from milk through hard dough stages, and stink bugs were collected from various pod filling stages of soybean. Insects were captured a day before infestation on caged cotton bolls. All insects were held individually in plastic portion cups for a 24 h fasting period and inspected to confirm that only healthy adults were used for infesting the caged cotton.

The experiment was conducted in 2013 and 2014 at the Texas A&M AgriLife Research and Extension Center farm in Corpus Christi, TX. Phytogen 367 WRF (Dow

AgroSciences, Indianapolis. IN) cotton seed was planted in early May on 91-m rows and 96-cm row centers at a field site of ≈0.4 ha, resulting in a plant stand of ≈77,800 plants per ha (31,500 plants per acre). Cotton plots were grown without irrigation in 2013 when 235 mm of rainfall was seen from April 15 to August 1 (National Weather Service 2019). Supplemental irrigation was provided by a drip system in 2014 (drought year) to attain a total of 241 mm of water inputs from April 15 to August 1 (Glover et al. 2019). Thiamethoxam insecticide (Centric, Syngenta Crop Protection, Greensboro, NC) was applied at labelled rates ca. every 10 days to maintain plots pest free before and after infestation. Thiamethoxam application was discontinued 14 days prior to infesting with experimental insects, and application was restarted at the conclusion of the infestation period. Other agronomic practices were normal for the region (Morgan 2018).

Single Boll Cage Experimental Design and Infestation

Insects were released into individually caged cotton bolls for a one-week period to characterize the effects of species and boll age on fruit retention, cotton boll injury, cotton boll rot, and yield. Experiments conformed to a species (including a no insect control) by boll age factorial. In 2013, available species were the verde plant bug, redbanded stink bug, brown stink bug, and green stink bug and boll ages were 0 and 3-day old post-anthesis. In 2014, species available were verde plant bug, brown stink bug, and green stink bug, and boll ages were 3, 5, and 7-day old post-anthesis. The boll age range increased in the second year based on first year date that showed a high amount of fruit abscission for newly set fruit (0-day old bolls). Treatment combinations of species and boll ages were

replicated 12 times in 2013 and 14 times in 2014, and each replication was set out in randomized blocks in the uniform cotton planting.

The experimental bolls were prepared for infestation by enclosing white blooms during the second week of bloom characterized as 10-12 NAWF (mid-bloom) from the first fruiting position (Kerby et al. 2010) with small organza fabric cages (12 by 13 cm, ~240 micron mesh, JoAnn's Fabrics, Hudson, OH) that protected bolls from feeding (Armstrong et al. 2005). Four days before each infestation, plants were hand sprayed to run-off with a short-residual U.V. sensitive pyrethrin insecticide (0.02% by volume, Bonide products, Oriskany, NY) to remove aphids and other small insects that may contaminate the caged cotton.

Cotton bolls of specific ages defined as 0- and 3-day old bolls post-anthesis in 2013, and 3, 5, and 7-day old bolls post-anthesis in 2014, were infested with each species individually, along with uninfested controls. To identify boll age, first position cotton bolls were identified at white bloom by tagging the boll pedicle with a plastic tag indicating the date and a colored ribbon was tied to the corresponding node of the main stem to identify plants with tagged bolls. First position cotton bolls were used for uniformity and because they are a significant portion of the total yield (Jenkins et al. 1990). Bolls were tagged every one to two weeks and maintained insect free with pyrethrin insecticide to ensure availability of clean developing bolls when the experiment was conducted.

Each cage was infested with one adult for each species for 7 days, along with maintaining a uninfested control. The seven-day infestation period was chosen to reflect a commercial field were insects might go undetected during weekly scouting. At the

conclusion of the infestation period a sampling of cages distributed across all species and both years ($n > 50$) were inspected for active insects, which were observed inside all cages. All caged treatments were then treated with thiamethoxam insecticide on day seven post-infestation and again on day 14 to eliminate non-target pest damage and remnant treatment insects including nymphs emerging from eggs laid by the adults.

Plant Injury and Yield Measurements

Cages remained in place until bolls fully matured and opened to expose lint, allowing bolls and potential cotton boll rot introduced by these insects to mature. Hand harvest occurred early August in 2013, and late August in 2014. At harvest, all caged bolls were rated for fruit retention, boll injury, cotton boll rot, and yield. For each treatment combination of species and boll age, mean percent fruit retention was calculated. All bolls retained on the plant were brought to the laboratory. Mean boll injury was calculated by first rating each boll using a boll injury scale. The scale ranged from 0 (representing no locule injury) to 1-3 (representing a progression of seed and lint degradation occurring in 1-3 locules, respectively) to 4 (representing severe degradation of seed and lint in all locules) (Brewer et al. 2013, Glover et al. 2019). Next, the boll interior was thoroughly inspected for symptoms of cotton boll rot (Medrano et al. 2009). Bolls were scored on presence or absence of cotton boll rot visually to obtain the percent bolls showing signs of boll rot. Yield data were estimated by cotton lint weights. To obtain lint weight, seed cotton was ginned by hand using a 10-saw laboratory cotton gin (Continental Eagle Crop., Prattville, AL). Weights were recorded as g weight of lint per boll.

Data Analysis

Before analyses, percent fruit retention and percent bolls with symptoms of cotton boll rot were transformed by arcsine square-root transformation of the proportion because of the wide range of values encountered (Neter et al. 1985). Back-transformed means were presented for ease of interpretation. Separate analyses were conducted for each measurement and each year because of the different species and boll ages used. The two-way factorial (species and boll age) were set out in physical blocks of replications. The SAS package (Proc GLM, Littell et al. 1991) which included the following specifications. The species and boll age factors were set as fixed effects; therefore, the residual was set as the error term for the main effects and the boll age-species interaction (Neter et al. 1985). If the interaction was significant, means separation analyses for the species and boll age main effects were conducted separately when the treatment levels were greater than two (Quinn and Keough 2002). If the interaction was not significant, the same means separation were used to compare means across species and boll age main effects. The means separation procedure used was Tukey's honest significant difference test ($\alpha = 0.05$) implemented in Proc GLM (Littell et al. 1991).

Results and Discussion

There was good evidence that the individual boll cages were successful in restricting feeding to and not disrupting feeding by the insects introduced into the cages. An ANOVA was run for each year because species used and boll ages caged varied across

years. In general, decreases in fruit retention, increases in cotton boll injury and cotton boll rot, and subsequent declines in lint weight resulted from the infestation of verde plant bug, brown stink bug, and green stink bug were compared to uninfested bolls and bolls infested with redbanded stink bug (Figures 3.1 and 3.2) (Table 3.1). All analyses below focus on significant species-boll age interactions and the main effects of species and boll age especially when it helps interpret the interaction or the interaction was not significant.

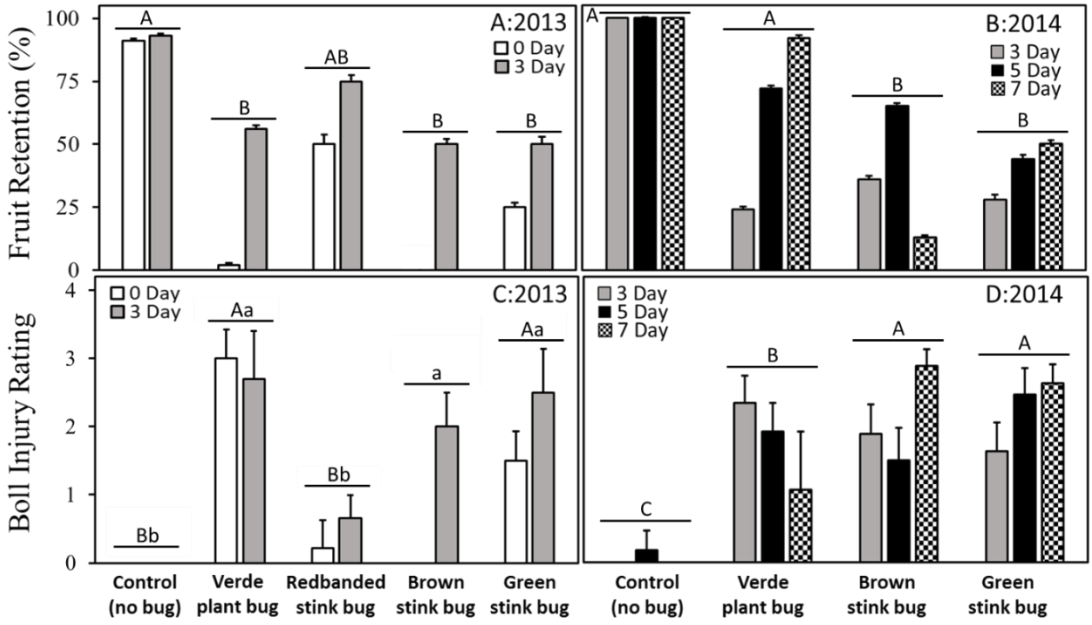

Figure 3.1. Mean Percent Fruit-retention and Boll Injury Rating. Mean (±SEM) percent fruit retention (A; 2013 and B; 2014); and boll injury rating (0-4) (C; 2013 and D; 2014) when selected species of stink bugs and a plant bug were infested individually on caged cotton bolls of several ages for a one-week period during mid-bloom. Bars with different letter(s) denote significant differences in species from their respective controls at $P < 0.05$; analyzed by Tukey's post-hoc test. See legend for boll ages used each year.

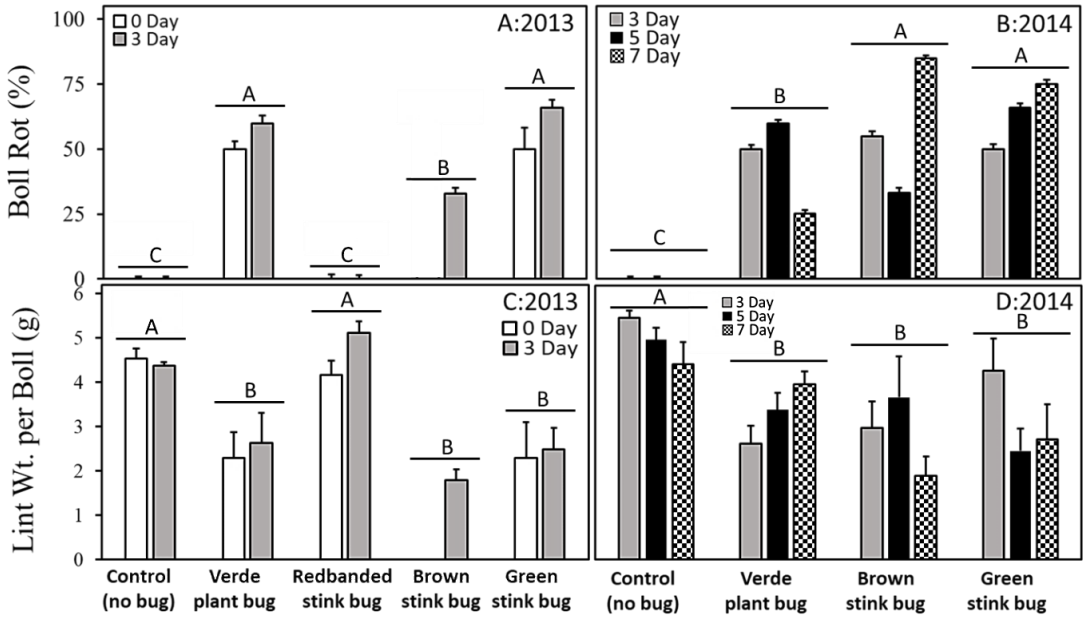

Figure 3.2. Mean Percent Boll Rot and Lint Weights. Mean (±SEM) percent boll rot (A; 2013 and B; 2014); and lint weights per boll (C; 2013 and D; 2014) when selected species of stink bugs and a plant bug were infested individually on caged cotton bolls of several ages for a one-week period during mid-bloom. Bars with different letter(s) denote significant differences in species from their respective controls at $P < 0.05$; analyzed by Tukey's post-hoc test. See legend for boll ages used each year.

Table 3.1. ANOVA Significance Results for Single Boll Cages. ANOVA significance tests of species, boll age, and their interaction when selected species of stink bugs and a plant bug were infested individually on caged cotton bolls of several ages for a one-week period during mid-bloom (2014, 2015).

	Fruit Retention		Boll Injury		Boll Rot		Yield	
Factor [a]	2013	2014	2013	2014	2013	2014	2013	2014
Species	F = 10.98 df = 4, 123 ***P* = 0.0001**	*F* = 7.91 df = 3, 187 ***P* = 0.0001**	*F* = 16.93 df = 4, 123 *P* = 0.0001	*F* = 20.86 df = 3, 187 ***P* = 0.0001**	*F* = 3.80 df = 4, 123 ***P* = 0.012**	*F* = 16.10 df = 3, 187 ***P* = 0.0001**	F = 5.82 df = 4, 123 ***P* = 0.0013**	*F* = 13.21 df = 3, 187 ***P* = 0.0019**
Boll age	*F* = 4.32 df = 1, 123 ***P* = 0.041**	*F* = 2.87 df = 2, 187 *P* = 0.098	*F* = 13.63 df = 1, 123 *P* = 0.0038	*F* = 2.76 df = 2, 187 *P* = 0.068	*F* = 4.42 df = 1, 123 ***P* = 0.043**	*F* = 2.18 df = 2, 187 *P* = 0.11	*F* = 1.51 df = 1, 123 *P* = 0.22	*F* = 4.98 df = 2, 187 ***P* = 0.0088**
Boll age - Species	F = 0.48 df = 4, 123 *P* = 0.74	*F* = 1.85 df = 6, 187 *P* = 0.094	*F* = 2.98 df = 4, 123 ***P* = 0.046**	*F* = 0.91 df = 6, 187 *P* = 0.48	*F* = 2.08 df = 4, 123 *P* = 0.12	*F* = 0.96 df = 6, 187 *P* = 0.45	F = 1.97 df = 4, 123 *P* = 0.13	*F* = 0.88 df = 6, 187 *P* = 0.51

Exact probabilities (*P*) are given, bolding is used to focus attention on significant results of the interaction, $P < 0.05$, or significant main effects when the interaction is not significant. The residual was used as the error term.

[a] Species were verde plant bug, redbanded stink bug, brown stink bug, and green stink bug in 2013, verde plant bug, brown stink bug, and green stink bug in 2014; Boll ages were 0 and 3-day old in 2013, and 3, 5, and 7-day old in 2014. The boll age – species interaction follows.

Fruit Retention

In 2013 and 2014, a significant boll age-species interaction was not detected ($P > 0.05$) (Table 3.1) (Figures 3.1A and 3.1B), but fruit retention differed across species (including the uninfested control) (Table 3.1) (Figures 3.1A and 3.1B). A Tukey's test ($\alpha = 0.05$) indicated that the verde plant bug, brown stink bug, and green stink bug caged bolls resulted in significantly lower fruit retention compared to bolls caged with redbanded stink bug and controls (bolls caged with no insect) in 2013 averaging across boll age (Figure 3.1A). Similarly, in 2014, a Tukey's test ($\alpha = 0.05$) indicated brown stink bug and green stink bug infested bolls resulted in significantly lower fruit retention compared to fruit retention for bolls caged with the verde plant bug and control bolls (Figure 3.1B).

In 2013, marginally significant boll age main effects were detected ($P = 0.041$), but not in 2014 ($P < 0.05$) (Table 3.1). In 2013, 0-day old bolls had lower fruit retention compared to older 3-day old bolls averaged across species (Figure 3.1A). Brewer et al. (2012a) found lower fruit retention when younger 0-day old bolls were caged with the verde plant bug. In data reported here, younger 0-day old bolls when caged with verde plant bug experienced up to 96% fruit abscission, and 100% fruit abscission when caged with the brown stink bug (no bolls retained) (Figure 3.1A). Similarly, Glover et al. (2019) showed significant decreases in fruit retention of younger bolls resulting from the infestation of verde plant bug at mid-bloom. To compensate for excessive declines in fruit retention the 0-day old boll age treatment was not repeated in 2014 and two additional boll ages were considered.

Boll Injury

In 2013, both the species-boll age interaction and main effects were significant (Table 3.1) (Figure 3.1C). Looking across species at 3-day old bolls, a Tukey's test ($\alpha = 0.05$) indicated bolls caged with verde plant bug, brown stink bug, and green stink bug were more injurious than bolls infested with the redbanded stink bug or control bolls (Figure 3.1C). These differences were less clear with comparing boll injury to 0-day old bolls across species (Figure 3.1C). Inspecting the interaction, 0-day old control bolls caged with no bug (no injury observed) and bolls caged with redbanded stink bug experienced minimal boll injury ($> 0.2 \pm 0.41$ injured locules per boll) compared to bolls infested with green stink bug (1.5 ± 0.38), and verde plant bug infested bolls which on average had the greatest boll injury score of (2.8 ± 0.43) injured locules per boll (Figure 3.1C). All 0-day old bolls caged with the brown stink bug in 2013 abscised (0% fruit retention, Figure 3.1A), and subsequent boll measures were not collected. Three day old control bolls had no damage and bolls caged with redbanded stink bug experienced minimal boll injury ($> 0.6 \pm 0.42$ injured locules per boll) compared to bolls infested with brown stink bug (2.0 ± 0.21), green stink bug (2.4 ± 0.38), and verde plant bug (2.6 ± 0.19) infested bolls (Figure 3.1C).

In 2014, the interaction was not significant ($P > 0.05$), boll injury differences were detected across species, but not boll age ($P > 0.05$) (Table 3.1) (Figure 3.1D). A Tukey's test ($\alpha = 0.05$) indicated insect infested bolls experienced significantly greater boll injury when compared to uninfested control bolls (Figure 3.1D). Verde plant bug (1.0 ± 0.41)

was less injurious compared to brown stink bug (2.0 ± 0.33) and green stink bug (2.3 ± 0.39) when averaged across boll age (Figure 3.1D) in 2014.

Overall, verde pant bug and stink bug infested plants consistently experienced increased boll injury when compared to uninfested plants, and more variation in boll injury was associated with species differences than boll age. The redbanded stink bug was less damaging to bolls when compared with the other stink bugs and verde plant bug. However, bolls infested with the redbanded stink bug experienced decreased fruit retention and boll injury distinct from control bolls. While boll injury was more variable across boll age than previously found, these results were consistent with reports of verde plant bug injury occurring primarily on younger bolls (Brewer et al. 2013). Additionally, Glover et al. (2019) reported boll injury rates similar to rates observed in this study, further supporting the susceptibility of younger less mature fruit.

Cotton Boll Rot

When visually inspecting open bolls at harvest, up to 66% of bolls had symptoms of cotton boll rot in 2013 and 85% in 2014, and variability was detected primarily across species (Table 3.1) (Figures 3.2A and 3.2B). Cotton boll rot reported here was significantly higher than previous findings of occurrence and magnitude of cotton boll rot symptoms when bolls were exposed to verde plant bug feeding (Brewer at al. 2012b). A significant species-boll age interaction was not detected both years ($P > 0.05$); however, species differences in occurrence of cotton boll rot were detected (Table 3.1). In 2013, a Tukey's test ($\alpha = 0.05$) indicated that bolls caged with verde plant bug, brown stink bug,

and the green stink bug had significantly more cotton boll rot than bolls caged with redbanded stink bugs and control bolls (Figure 3.2A). Cotton boll rot was never detected in bolls caged with redbanded stink bug or control bolls (Figure 3.2A). These results are inconsistent with Glover et al. (2019) who reported relatively low incidence of cotton boll rot (no greater than 10%) when caging whole cotton plants during mid-bloom with the redbanded stink bug, while cotton boll rot incidence was much higher in cages infested with other stink bug species. The lack of boll rot observed in bolls caged with the redbanded stink bug and the relatively high incidence of cotton boll rot for other species in the current study may be associated with differences in habitat reservoirs for the disease (redbanded stink bug was collected in soybean, while the other species were collected in sorghum) or the efficiency in transmitting the disease that is occurring to some degree across species.

In 2014 a Tukey's test ($\alpha = 0.05$) indicated that bolls caged with the brown stink bug and green stink bug experienced significantly more cotton boll rot (up to 85%) compared to bolls infested with the verde plant bug (up to 62%). Cotton boll rot was never detected in the control bolls (Figure 3.2B). In 2013, a marginally-significant boll age main effect was detected ($F = 4.42$; df = 1, 123; $P = 0.043$) (Figure 3.2A) but was not ($P > 0.05$) (Table 3.1) in 2014. Detailed cross-species disease transmission and vector competency studies may help explain what drives the variation detected across species in this study.

Lint Weight

In 2013 and 2014, the species-boll age interaction was not significant ($P > 0.05$) (Table 3.1) (Figures 3.2C and 2.3D). In 2013, lint weight varied across species but not across the two boll ages (Table 3.1) (Figure 3.2C). A Tukey's test ($\alpha = 0.05$) indicated a significant decline in lint regardless of boll age when exposed to verde plant bug, brown stink bug and green stink bug compared to the other species and the uninfested controls. Bolls infested with redbanded stink bug had lint weight comparable to the uninfested bolls, suggesting boll response to herbivory from this species may be less severe and may be associated with the low levels of cotton boll rot seen for this species (Figure 3.2). Lint depression on a per boll basis reflected boll injury and cotton boll rot observed here (Figures 3.1A, 3.1C and 3.2A), as well as that observed in whole plant cage studies except for redbanded stink bug (Glover et al. 2019). Although the interaction was not significant in the 2014, both species and boll age main effects were detected (Table 3.1). In 2014, a Tukey's test ($\alpha = 0.05$) indicated significant lint decline when infested with any species compared to infested bolls (Figure 3.2D). In comparison, differences in yield detected across boll age were not readily apparent (Figure 3.2D).

In this 2-year study, the same species were considered as used in Glover et al. (2019) in order to examine the extent that boll age sensitivity to feeding and species differences contributed to differences observed in specific damage and yield when cotton was infested at mid-bloom. Differences in fruit retention, boll injury, boll rot, and yield were detected across species in 2013 and 2014. The contrast of results comparing 0, 3, 5, and 7-day old bolls reflects past studies which showed that verde plant bug readily feeds

on large squares and small bolls <10-day old (Brewer et al. 2012a) and is an important contributor to decreased fruit retention. In contrast, southern green stink bugs can continue feeding on larger bolls (Greene et al. 2001). From a management viewpoint, this may have implications on the window of field monitoring that is needed when sampling for these insects. The decline in fruit retention and increased boll injury (Figure 3.1) support the interpretation that main causes of yield decline was poor fruit retention and boll injury that led to cotton boll rot (Brewer et al. 2013, Glover et al. 2019). The variation observed in frequency and magnitude of cotton boll rot in this two-year study suggest a seasonality in the presence of boll rot pathogens and or potential differences in transmission efficiency cross species. Further research to define species-specific disease relationships would be valuable, particularly because it appears to be an important driver of yield decline (see lack of cotton boll rot and yield decline for redbanded stink bug here [Figures 3.2A and 3.2C]).

From a management viewpoint, the similarities in fruit retention, boll injury, cotton boll rot and subsequent yield decline observed in these experiments further explains and supports the mid-bloom period of cotton development as containing the largest array of susceptible boll ages, and the more sensitive growth period. Furthermore, the narrow range of yield depression observed in these experiments across stink bug and plant bug infested bolls supported the construction of a common economic injury level for at two species of stink bugs when a mixed species complex is present (Glover et al. 2019).

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CHAPTER IV

TRANSMISSION OF COTTON SEED AND BOLL ROTTING BACTERIA BY THE VERDE PLANT BUG

Introduction

The verde plant bug, *Creontiades signatus* (Distant) (Hemiptera: Miridae), is a significant cotton pest in south Texas and has been associated with cotton seed and boll rot, commonly called cotton boll rot (Brewer et al. 2012a). Verde plant bug was a suspected vector of the disease based on similar insect-disease relationships previously documented for stink bugs (Medrano et al. 2007). Armstrong et al. (2009) isolated bacteria associated with verde plant bug feeding injury on cotton bolls. Medrano et al. (2014) identified the bacteria *Serratia marcescens* as a boll rot pathogen isolated from bolls fed upon by verde plant bug. Currently, management for verde plant bug in south Texas assumes that the boll rot pathogen is present and the insect is capable of transmitting the pathogen. Economic thresholds for verde plant bug have been established that assumed presence of boll rot pathogens (Glover et al. 2019). The potential of economic damage is evaluated by checking for signs of internal feeding, including opening green bolls during field monitoring activities (Greene et al. 2001, Brewer et al. 2013).

Unfortunately, early detection of cotton boll rot infection caused by *S. marcescens* and possibly vectored by verde plant bug is limited since there is an absence of external infection symptoms on immature green bolls, and likely no visible internal symptoms soon

after infection as seen by boll rot bacteria vectored by stink bugs (Medrano et al. 2007). Known disease symptoms from cotton boll rotting bacteria introduced by stink bugs include discolored lint and necrotic seed that can be observed only when infected green bolls are cross-sectioned at least one week after initial infection or once mature bolls open (Medrano et al. 2007). If transmission potential can be authenticated, then insecticide use decision-making based on economic injury levels and verde plant bug monitoring (Glover et al. 2019, Brewer et al 2013) can be revisited and adjusted based on transmission and retention risk.

We investigated verde plant bug as a cotton boll rot vector to expand our previous work that showed an association of verde plant bug with bacterial rot (Brewer et al. 2012) and showed *Serratia marcescens*, isolated from bolls feed upon by verde plant bug, can cause the disease (Medrano et al. 2014). The specific objectives of this study were to confirm pathogenicity and transmission of bacteria recovered from bolls fed on by verde plant bug exposed to the bacteria *Serratia marcescens* and examine disease retention as verde plant bug feeds on multiple bolls in a field setting.

Materials and Methods

Individual cotton bolls were infested with verde plant bug exposed and not exposed to the boll rot causing bacterial pathogen *Serratia marcescens* in experiments conducted in 2016 and 2017 at the Texas A&M AgriLife Research and Extension Center farm in Corpus Christi, TX. Phytogen 333 WRF (Dow AgroSciences, Indianapolis. IN) cotton

seed was planted in late April on 91-m rows and 96-cm row centers at a field site of ≈0.6 ha, resulting in a plant stand of ≈77,800 plants per ha (31,500 plants per acre). Cotton plots were grown under dryland conditions. Thiamethoxam insecticide (Centric, Syngenta Crop Protection, Greensboro, NC) at labelled rates was used ca. every 10 days to maintain plots pest free before infestation. Thiamethoxam application was discontinued 14 days prior to infesting with experimental insects. Other agronomic practices were normal for the region (Morgan 2018).

Exposure of Insects to S. marcescens

Verde plant bug adults used to infest caged cotton bolls were taken from a laboratory colony established from field collections. The laboratory colony was established from adult verde plant bugs collected from several wild and cultivated host plants, including sorghum, soybean and several seepweeds, *Suaeda* spp. (Chenopodiaceae) (Armstrong 2010), along the Texas Gulf coast. Insects were collected using a modified leaf blower that displaces insects from vegetation and transfers them into an inflatable sock that fits on the opposite end of the blower's fanned nozzle, known as a KISS-sampler (keep it simple sampler) (Beerwinkle et al. 1997). Verde plant bugs were collected in 2016 from a mixture of seepweeds and grain sorghum from milk through hard dough stages, and in 2017 on soybean from flowering through full pod development. All insects were inspected to confirm only healthy adults were used to establish the laboratory colony. Insects were reared under laboratory conditions on a diet of sanitized (bleached) green beans, *Phaseolus vulgaris* L. Colony adults and immature stages were fed fresh

green bean pods thoroughly rinsed in a 5% sodium hypochlorite solution and dried. The beans were replaced at 2-3d intervals and all life stages were held in a ventilated plastic container at 28˚C and a photoperiod of 14:10h (L:D) following the protocol of Medrano et al. (2007, 2016). The colony was kept for three generations to purge to the extent possible boll rot causing bacteria that may have been present on field collected verde plant bugs used to establish the colony.

To prepare for infecting verde plant bug with the bacteria for experimental purposes, a Rif resistant bacterial mutant *S. marcescens* strain (CC119-R), was maintained on Luria Bertani agar (LBA; Difco, Detroit, MI) amended with Rifampicin antibiotic with levels up to 200 µg ml^{-1} and incubated at 28˚C (Medrano et al. 2014). The strain was derived from cotton bolls infested with verde plant bug and associated with cotton boll rot from the same location of the field experiment (Brewer et al. 2012). Bacteria isolated from the bolls were identified as *S. marcescens* on the basis of phenotype testing, fatty acid profiling (similarity index = 0·94), and 16s ribosomal DNA sequence analysis (99% nucleotide identity) and were shown to cause disease on cotton bolls (Medrano et al. 2014) comparable to field observed disease. Fresh green beans were sterilized, cross-sectioned into ≈3cm pieces, and soaked for 2 min in either the disease-causing Rif resistant bacteria (CC119-R) inoculum or in sterile water. The bean pieces were then blotted dry using sterile paper towels as described in Medrano et al. (2007, 2016). A treated bean section was aseptically transferred into a sterile disposable Petri dish along with a single teneral third-generation verde plant bug from the colony. Multiple dishes were set up depending on the needs of the experiment. After two days of incubation with the exposed bean piece

each verde plant bug was transferred using a sterile aspirator (i.e. ethanol dipped and then flamed) into a new sterile petri dish containing a sanitized (bleached) non-exposed green bean. The process of feeding on sterile water soaked green beans was repeated for another two days in order to minimize *S. marcescens* exposure to the plant bug body surface. Finally, the verde plant bugs were aspirated into one-ounce plastic portion cups and held for a 24h fasting period before being used in 2016 and 2017 field experiments. Concurrently, another set of verde plant bugs fed on non-exposed green beans (i.e. green beans provided to insects soaked for 2 min in sterile water) and processed through the same bean replenishment and fasting period before being used as the non-exposed verde plant bug in the same field experiments.

At infestation, randomly selected adults from the exposed and non-exposed treatment groups from the colony were placed in one-once portion cups and released mid-morning on individual bolls enclosed in single boll exclusion cages. The effects of *S. marcescens* exposed and non-exposed verde plant bugs, in addition to controls (bolls caged with no bug) on boll injury and cotton boll rot were measured and verified using molecular techniques.

Single Boll Cage Experimental Design and Insect Infestation

In 2016 to examine initial pathogenicity and transmission of *S. marcescens*, individual verde plant bugs exposed and non-exposed to *S. marcescens* (CC119-R), along with a no insect control, were released into individual caged cotton bolls of two ages for a one-week period. In 2017, the experiment was repeated, and immediately followed by a disease

retention study by moving surviving verde plant bugs exposed to CC119-R, from the initial cages to a new set of previously caged insect and disease-free bolls for a 24 h period to examine the retention of the bacteria for potential transmission into additional bolls.

The experimental bolls were prepared for infestation by enclosing white blooms during the third second of bloom characterized as 10-12 NAWF on the first (mainstem) fruiting position (Kerby et al. 2010) with small organza fabric cages (12 by 13 cm, ~240 micron mesh, JoAnn's Fabrics, Hudson, OH) that protected bolls from feeding (Armstrong et al. 2005). Four days before each infestation, plants were hand sprayed to run-off with a short-residual U.V.-sensitive pyrethrin insecticide (0.02% by volume, Bonide products, Oriskany, NY) to remove aphids and other small insects that may contaminate the caged cotton bolls.

In 2016 and 2017 initial pathogenicity and transmission field studies, the experimental design conformed to a disease exposure treatment by boll age factorial. The disease exposure treatments were verde plant bug non-exposed to CC119-R, verde plant bug exposed to CC119-R, and a no insect control. The boll age treatments were 5 and 7-day old bolls post-anthesis. A minimum of 65 replications per treatment combination were conducted across both years. A 24 h infestation period in 2017 simulated the risk of allowing these insects to persist in the field, retain the disease, and transmit the disease to more than one boll. A 7-day infestation period in 2016 was chosen to reflect a commercial field were insects might go undetected during weekly scouting.

In the 2017 disease retention study, surviving insects from bolls caged with verde plant bug exposed to CC119-R from the 2017 initial pathogenicity and transmission

experiment were aspirated from their respective bolls after the prescribed 24 h infestation period and immediately re-caged on additional cotton bolls (6 and 8-day old bolls) to investigate if insects were able to continue to transmit disease (disease retention) to multiple bolls after disease acquisition. In 2017, the disease retention study conformed to randomized complete block of the boll age treatment (6 and 8-day old bolls post-anthesis) with a minimum of 35 replications for each boll age. At the conclusion of the infestation period of each experiment in each year a sampling of caged treatment bolls ($n > 50$) were inspected for active insects, which were observed inside all cages inspected.

Cotton bolls of specific ages were defined as 5 and 7-day old bolls post-anthesis in 2016 and 2017 initial pathogenicity and transmission, and 6 and 8-day old bolls post-anthesis in 2017 disease retention study. Bolls were identified at white bloom by tagging the boll pedicle with a plastic tag indicating the date and a colored ribbon was tied to the corresponding node of the main stem to identify plants with tagged bolls. First position cotton bolls were used for uniformity and because they are a significant portion of the total yield (Jenkins et al. 1990). Bolls were tagged every 1 to 2 weeks and maintained insect free with pyrethrin insecticide to ensure availability of clean developing bolls when the experiment was conducted. At the end of the infestation period of 7 days or 24 h depending on the experiment, the caged bolls were then treated with thiamethoxam insecticide and again 7 days later to eliminate non-target pest damage and remnant treatment insects including nymphs emerging from eggs laid by the adults.

Cotton Boll Evaluation

Caged bolls remained on the plants for two weeks after the infestation allowing any *S. marcescens* introduced by verde plant bug to express cotton boll rot disease symptoms. At this time, additionally caged bolls with *S. marcescens*-exposed verde plant bugs were opened and the interior were visually inspected for cotton boll rot. Disease symptoms were photographed in the laboratory. All remaining caged green cotton bolls were transported to the USDA-ARS, College Station, TX, where they were evaluated using visual and molecular techniques (Glover et al. 2019, Medrano et al. 2007, 2016).

Briefly, visual examination of locules from collected bolls were scored for boll injury using a rating on a scale ranging from 0 (representing no locule injury) to 1-3 (representing a progression of seed and lint degradation occurring in 1-3 locules, respectively) to 4 (representing severe degradation of seed and lint in all locules) (Brewer et al. 2013a). Bolls were visually inspected for presence or absence of symptoms of cotton boll rot in each locule (Medrano et al. 2009) scoring the number of locules with boll rot presence (0 to 4) scale. Methods and parameters reported in Medrano et al. (2007, 2016) were used for microbiological assessment. Briefly, bolls were surface sterilized, diseased and asymptomatic locule tissue were aseptically harvested, and bacterial concentrations of the tissues were recorded using standard microbiological methods to isolate diseased tissues on bacteriological media. Luria Bertani agar amended with Rifampicin antibiotic (200 ug/ml) was used to screen for CC119-R.

Macerated boll tissue samples were spread and incubated at 37˚C for 24 hours on the rifampicin amended plates and scored for presence or absence of growth of bacterial strain CC119-R.

Data Analyses

All measurements taken from the caged treatments were used to examine treatment effects by using an ANOVA for a two-way factorial (disease exposure and boll age factors) in the 2016 and 2017 initial pathogenicity and transmission field experiments, and an ANOVA for a randomized complete block (boll age) in the 2017 disease retention field study (Proc GLM, Littel et al. 1991). The disease exposure and boll age factors were set as fixed effects; therefore, the residual was set as the error term for the main effects and the disease exposure-boll age interaction for the factorial (Neter et al. 1985). If the interaction was significant, means separation analyses for the species and boll age main effects were conducted separately when the treatment levels were greater than two (Quinn and Keough 2002). If the interaction was not significant, the same means separation test was used to compare disease exposure treatments averaged across boll age. The means separation procedure used was Tukey's honest significant difference test ($\alpha = 0.05$) implemented in Proc GLM (Little et al. 1991).

Percent laboratory plated and confirmed cotton boll rot samples (CC119-R) were transformed by arcsine square-root transformation of the proportion before analysis to compensate for potential deviation from normality (Neter et al. 1985). Back transformed percentages were presented for ease of interpretation. Measurements were analyzed

separately in 2016 and 2017 initial transmission experiments and in the 2017 disease retention study.

Results

Confirming Exposure of Insects to S. marcescens

A preliminary analysis using approximately 35 verde plant bugs exposed and non-exposed were used to confirm the effectiveness of the dietary regime for infecting verde plant bug with the Rif-resistant *S. marcescens* bacteria (CC119-R), known to cause cotton boll rot. Bacteria were isolated from all insects exposed to Rif-resistant bacteria via the dietary treatment placed on discriminating media. Concentrations of Rif-resistant bacteria and ranged from 10^6 to 10^8 CFUs per insect. Rifampicin-resistant bacteria were not recovered from non-exposed bugs that were used as negative controls (i.e., not exposed to CC119-R). The highest concentration of rifampicin-resistant bacteria ranged from 10^3 to 10^8 CFU's per insect from verde plant bugs exposed to CC119-R (Table 4.1) that were taken from a subset of bugs used in the field experiment.

For the field transmission study, cotton boll injury and boll rot from the infestation of verde plant bug exposed to CC119-R were significantly higher than non-infested and non-exposed to CC119-R treatments. The bacterial infection process did not apparently affect insect vigor based on average boll damage ratings (Figures 4.1A, 4.1B, and 4.1G) where similar feeding damage was observed across both exposed to CC119-R and non-exposed verde plant bugs.

Table 4.1. Bacterial Concentration Ranges. Bacterial concentration ranges from verde plant bug infested bolls that were exposed or non-exposed to Rif-resistant *Serratia marcescens* (CC199-R) known to cause cotton boll rot (2016, 2017).

	LBA [a]		LBA Rif [a]	
	Lowest-Highest Bacterial Concentration [b] 2016	Lowest-Highest Bacterial Concentration 2017	Lowest-Highest Bacterial Concentration 2016	Lowest-Highest Bacterial Concentration 2017
Boll age				
	----------Control----------			
5 d	1-6	1-9	Nd	Nd
7 d	2-7	2-7	Nd	Nd
	----------Non-exposed----------			
5 d	1-8	1-9	Nd	Nd
7 d	3-7	3-7	Nd	Nd
	----------Exposed----------			
5 d	2-7	1-9	1-7	1-8
7 d	3-7	2-8	3-7	1-6
	----------Exposed----------			
6 d		1-9		3-8
8 d		1-8		1-6

[a] Luria–Bertani agar; LBA amended with rifampicin (100 lg ml).

[b] Bacterial concentrations are expressed as log CFUs/g of cotton tissue; Nd, indicates no presence of Rif-resistant *Serratia marcescens* (CC199-R); Presence of (CC199-R) was only detected from bolls infested with verde plant bug exposed to Rif-resistant *Serratia marcescens*.

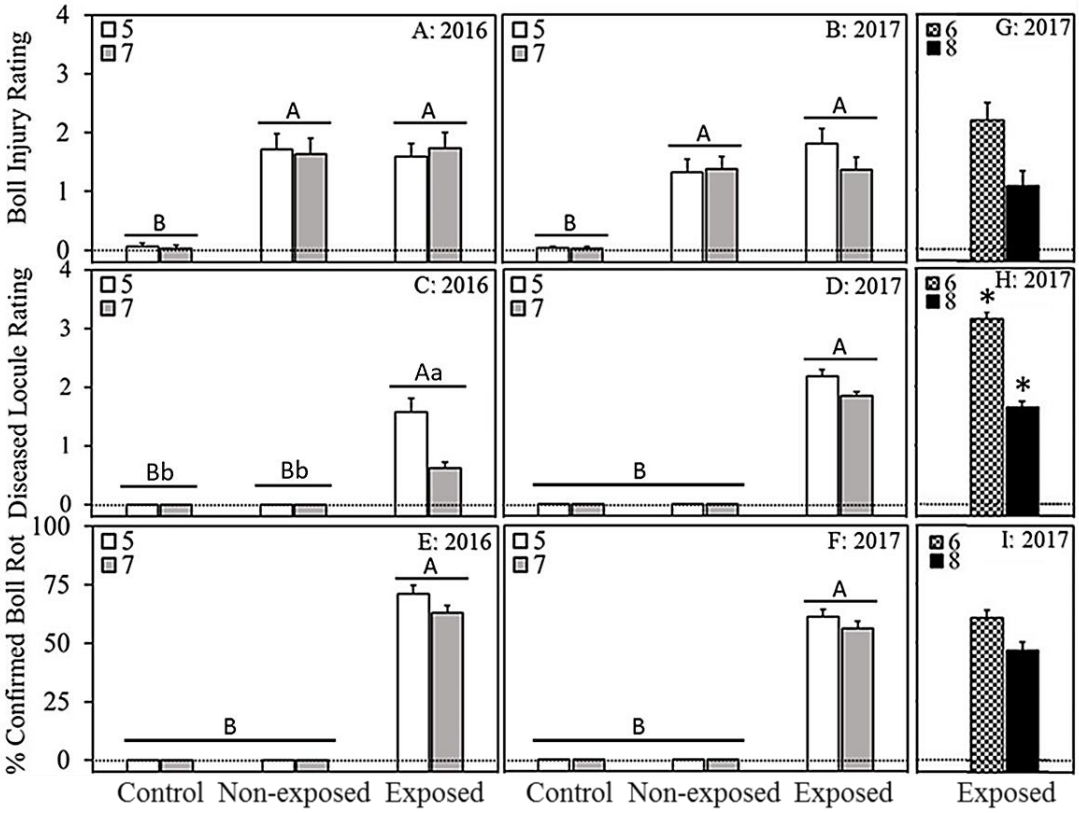

Figure 4.1 Mean Boll Injury, Diseased locules, and Confirmed Disease. Mean (±SEM) boll injury rating (0-4), diseased locules per boll rating (0-4), and percent plated and confirmed Rif-resistant *Serratia marcescens* (CC119-R) for initial pathogenicity and transmission field experiments (A: 2016 and B: 2017) (C: 2016 and D: 2017) (E: 2016 and F: 2017), and the disease retention study (G: 2017) (H: 2017) (I: 2017). Infestation duration was 1 wk in 2016 and 24 h in 2017. Bars with different letter(s) denote significant differences in disease exposure treatment from their respective controls at $P < 0.05$; analyzed by Tukey's post-hoc test. See legend for boll ages used each year.

Disease Symptoms, Transmission, and Confirmation

In 2016 and 2017 initial pathogenicity and transmission field experiments, damage to locule tissue from cracked green bolls caged with verde plant bug exposed to CC119-R was confined to the immediate area surrounding the puncture wound (Figure 4.2) Bacteria were not detected from carpel imprints on discriminating media (containing Rif) by using representative bolls after caging with insects (data not shown). From the bolls

analyzed (n > 400) for signs of insect feeding, 384 had puncture wounds on the inner carpel wall, had affected lint tissue, or both in 2016 and 2017. The number of punctures for all bolls caged with an insect ranged from 1 to 6 per boll with 83% of bolls having 1 to 5 lesions. Both pierced and uninjured locules were observed on individual bolls. Based on observations of lint and seed, 89% of the inner carpel lesions inflicted by verde plant bug not exposed to CC119-R had no corresponding locule tissue damage (Figure 4.2A, left panel). Conversely, all bolls that had been infested with verde plant bug exposed to CC119-R exhibited inner carpel damage and at least seed discoloration (Figure 4.2A, right panel). Bacteria were not detected from samples of lint or seed tissue from bolls caged with verde plant bug non-exposed to CC119-R or control bolls (Table 4.1). Disease symptoms consistently corresponded with the detection of CC119-R based on growth on the LBA media amended with rifampicin.

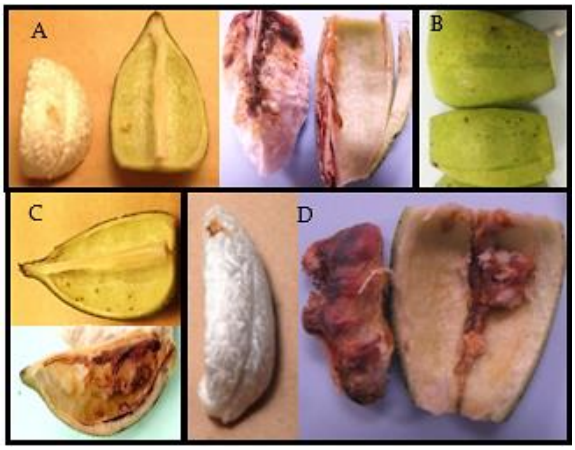

Figure 4.2. Internal and External Feeding Effects of Bolls Infested with Verde Plant Bug. Internal and external effects of feeding on field-grown cotton bolls caged with verde plant bug, *Creontiades signatus* (Distant) (Hemiptera: Miridae), exposed and non-exposed to Rif-resistant *Serratia marcescens* (CC119-R) after two weeks exposure. Panel A: left boll shows inner carpel and locule damage associated with verde plant bug non-exposed to CC119-R, the right boll was feed on by a verde plant bug exposed to CC119-R, illustrating initial inner carpel and locule damage. Panel B: effects of verde plant bug non-exposed to CC119-R feeding on the external carpel wall (top section) and verde plant bug exposed to CC119-R (bottom section) cannot be distinguished by boll inspection. Panel C: effects of verde plant bug non-exposed to CC119-R feeding on the internal carpel wall (top section) and verde plant bug exposed to CC119-R (bottom section) can be distinguished by cracking green bolls after at least two weeks after the initial infection. Panel D: effects of verde plant bug non-exposed to CC119-R feeding on developing lint (bottom left section) and verde plant bug exposed to CC119-R (bottom middle section) can be distinguished by inspecting open bolls near harvest.

Boll Injury

In 2016 and 2017, a significant disease exposure-boll age interaction was not detected ($P > 0.05$) (Figures 4.1A and 4.1B), but a significant disease exposure main effect was detected (2016: $F = 29.84$; df = 2, 114; $P < 0.0001$; 2017: $F = 40.67$; df = 2, 290; $P < 0.0001$). Overall, bolls caged with verde pant bug either exposed or not exposed to *S. marcescens*, consistently experienced higher boll injury compared to bolls caged without an insect. The bacterial infection process did not apparently affect insect vigor based on average boll damage ratings where similar feeding damage was observed across all bolls caged with verde plant bug (Figures 4.1A and 4.1B). In 2016 and 2017, significant boll

age main effects (interaction was not significant) were not detected ($P > 0.05$), supporting susceptibility of bolls to verde plant bug herbivory on bolls ranging from 5 to 7-days old post-anthesis.

Cotton Boll Rot

When visually inspecting green bolls two weeks after infestation, up to 62% of bolls infested with *S. marcescens*-exposed verde plan bug had symptoms of cotton boll rot in 2016 and 54% in 2017 (Figures 4.1C and 4.1D). These results were significantly higher than previous findings of occurrence and magnitude of cotton boll rot symptoms in field-collected bolls (Brewer at al. 2013). Disease symptoms were never detected in bolls caged with verde plant bug non-exposed or controls. In 2016, a significant disease exposure-boll age interaction was detected ($F = 17.63$; df = 2, 114; $P < 0.0001$), but was not significant in 2017 ($P > 0.05$) (Figures 4.1C and 4.1D) in the diseased locule rating measurement. The interaction reflected a greater tendency of cotton boll rot symptoms in younger bolls (5 day old) than older bolls, consistant with previous finding that boll rot was more severe when infestation occurred mid-bloom compared to late-bloom (Glover et al. 2019). Visual symptoms of locule rot were detected in bolls caged with verde plant bug exposed to CC119-R (Figure 4.2A, right panel; 5 day old boll, and Figure 4.2D, right panel; 7 day old boll). In 2017, looking at the main effects (interaction was not significant) significant disease exposure main effects were detected ($F = 45.20$; df = 2, 290; $P < 0.0001$), and signficant boll age main effects were not ($P > 0.05$). A Tukey's test ($\alpha = 0.05$) indicated bolls caged with verde plant bug exposed to CC119-R consistently experienced symptoms

of locule rot compared to bolls caged with verde plant bug non-exposed and control bolls (Figure 4.1D). Simliar to 2016 field experimetns, cotton boll rot was never detected in bolls caged with non-exposed verde plant bug or control bolls. In general, cotton boll rot seemed to track boll injury (Figures 4.1A, 4.1B, 4.1C, and 4.1D); as boll injury increased so did boll rot in 2016 and 2017 field experiments, with a tendency of greater cotton boll rot expression as boll age decreases. These data support the susceptibility of younger bolls to feeding damage and cotton boll rot (Brewer et al. 2012, Glover et al. 2019).

Laboratory Disease Confirmation

When visually inspecting plated tissues on discriminating media (media containing Rif) from control bolls, significant disease exposure-boll age interactions were not detected ($P > 0.05$) in 2016 or 2017 (Figures 4.1E and 4.1F), but a significant disease exposure main effect was detected (2016: $F = 5.80$; df = 2, 114; $P = 0.0040$; 2017: $F = 8.02$; df = 2, 155; $P = 0.0005$) (Figures 4.1E and 4.1F). In 2016 and 2017, a Tukey test ($\alpha = 0.05$) indicated bolls caged with verde plant bug exposed to CC119-R consistently experienced symptoms of cotton boll rot compared to bolls caged with verde plant bug non-exposed to CC119-R and uninfested controls where cotton boll rot was never detected (Figures 4.1E and 4.1F). In 2016 and 2017, when looking at the boll age main effect differences were not detected ($P > 0.05$), confirming susceptibly of 5 and 7-day old bolls to verde plant bug vectored boll rot (CC119-R). Overall, the rifampicin sensitive bacterial strain (CC119-R) were only recovered from bolls caged with verde plant bug exposed to

CC119-R, and never from controls (bolls caged with no bug) or bolls caged with verde plant bug non-exposed to CC119-R (Figures 4.1E and 4.1F).

Retention of S. marcescens by the Verde Plant Bug

In the 2017 disease retention study, from the bolls analyzed (n = 73) for signs of insect feeding, all 68 had inner carpel damage, affected lint tissue, and discolored seed and lint. The number of punctures for all bolls caged with an insect ranged from 1 to 7 per boll. All bolls caged with verde plant bug exposed to CC119-R exhibited inner carpel damage and seed discoloration (Figure 4.2A, upper right panel; Figure 4.2D, bottom right panel). Bacteria concentrations recovered on the selective medium ranged from10^1 to10^3 CFUs/g tissue (Table 4.1). Again, disease symptoms consistently corresponded with the detection of the Rif-resistant bacteria based on selective medium growth. Disease symptoms consistently corresponded with the detection of CC119-R based on discriminating medium growth.

In the 2017 disease retention field study, a significant boll age effect on boll injury and cotton boll rot were not detected ($P > 0.05$) (Figure 4.1G), but more diseased locules per boll were found in 6 day old bolls, with an average of 3.1 diseased locules, compared to older 8 day old bolls with an average of 1.6 diseased locules per boll ($F = 5.60$; df = 1, 68; $P < 0.021$) (Figure 4.1H). Up to 67% of bolls had symptoms of cotton boll rot by visual inspection (Figure 4.1H). When visually inspecting plated tissues on discriminating media (media containing Rif), up to 60% of the plated samples had confirmed cotton boll rot (CC119-R). This transmission rate compared to 54 to 62% in the initial transmission study,

demonstrating the ability of the verde plant bug to retain the disease and transmit cotton boll rot (CC119-R) successfully to an additional fruiting (bolls) sites with consistent efficiency across feeding on two bolls (Figure 4.1I).

Discussion

This study verified that providing insects green beans first immersed in a solution contaminated with the disease agent was an effective method for infesting insects with the cotton pathogen as reported by Medrano et al. (2007, 2016). The use of our disease model provided a method to systematically analyze insect-derived boll injury resulting from verde plant bug feeding/probing alone, and in tandem with boll infection by an insect-vectored pathogen at various stages of fruit development. Appearance of cotton boll rot infection symptoms ranged from reddening of the seed to seed and lint necrosis, and always corresponded with the detection of *S. marcescens* strain CC119-R. An additional set of bolls were caged with verde plant bug exposed to CC119-R and allowed to fully mature and open to photograph the range of disease expression at harvest (Figure 4.3).

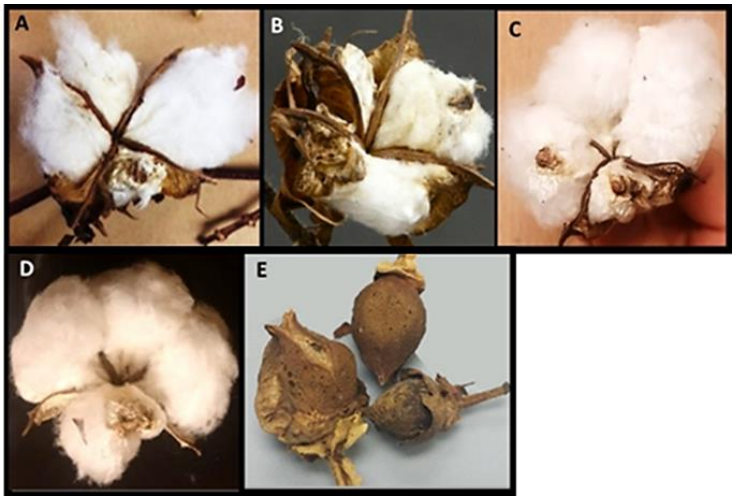

Figure 4.3. Disease Expression in Cotton Bolls Infested with Verde Plant Bug. Range of disease expression of cotton bolls caged with verde plant bug, *Creontiades signatus* (Distant) (Hemiptera: Miridae), vectoring Rif-resistant *Serratia marcescens* (CC119-R). Lint injury and positive visual symptoms of cotton boll rot after infestation with verde plant bug exposed to CC119-R on a five (A), six (B), seven (C), and eight (D) day old boll. Abscised fruit from bolls infested with verde plant bug exposed to CC119-R (E).

This study demonstrated boll injury caused by verde plant bug feeding as distinct from cotton boll infections by verde plant bug vectoring opportunistic *S. marcescens*. The symptoms of cotton boll rot and boll injury shown here were similar to those described in various reports of cotton seed and boll rotting disease (Medrano et al. 2016), and injury caused by verde plant bug infestations (Brewer et al. 2012). Medrano et al. (2007) working with the southern green stink bug, reported bacteria that included *Serratia*, *Staphylococcus*, *Pseudomonas*, *Pantoea*, and *Enterobacter* at levels reaching 10^9 CFUs/g tissue from locules with evidence of stink bug punctures. This study corroborated this earlier observation with similar bacterial concentrations from locules with feeding and probing indications by verde plant bug. All locules from non-infested control bolls were asymptomatic and no bacteria was detected ($<10^1$ CFUs/g tissue). Furthermore,

concentrations seemed to remain at or about the same level across the two boll ages considered here. This study authenticated that verde plant bug injury directly caused by piercing/sucking feeding was distinct from disease infection caused by introduction of *S. marcescens* through the feeding process. Results from these experiments indicated that damage associated with verde plant bug infestations of developing bolls was in large part dependent on whether the insect transmitted the cotton pathogen *S. marcescens*.

This study was the next logical step to expand our work which established decision-making procedures for verde bugs that assumed the presence of boll rot pathogens (Glover et al. 2019). Our disease transmission work with the insect vectors authenticate presence and persistence of the disease and risk of pathogen transmission by the vector in cotton fields. The nature of bacterial persistence within the insect as seen with the insect's ability to retain and transmit disease across two bolls may be useful for risk assessment information and for evaluating insect monitoring needs for verde plant bug.

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CHAPTER V

PHOTOPERIOD-SPECIFIC DISTRIBUTION OF THE GREEN STINK BUG (HEMIPTERA: PENTATOMIDAE) ON COTTON

Introduction

Injury to cotton, *Gossypium hirsutum* L. (Malvaceae), by stink bugs (Hemiptera: Pentatomidae) has increased substantially with the widespread adoption of transgenic Bt (*Bacillus thuringiensis)*-cotton cultivars targeting lepidopterans pests (Luttrell et al. 2015). The resulting insecticide use decline has likely released sucking bugs, including stink bugs, that were formerly controlled by broad-spectrum insecticides (Lu et al. 2010). Consistent with higher numbers of these pests, cotton boll injury due to stink bug feeding has increased substantially during the last two decades in the southern United States including Texas, China, and other cotton-producing counties (Lu et al. 2010, Greene et al. 2001, Luttrell et al. 2015, Glover et al. 2019).

The complex of stink bugs on cotton that frequently occur in south Texas include redbanded stink bug, *Piezodorus guildinii* (Westwood), brown stink bug, *Euschistus servus* (Say), southern green stink bug, *Nezara viridula* (Say), and the green stink bug, *Chinavia hilaris* (Say) (Hemiptera: Pentatomidae). Members of this boll-feeding complex are the same or representative of stink bugs found elsewhere in cotton (Greene et al. 2001). Historically, the southern green stink bug, brown stink bug, and the green stink bug are

known to be economic pests of cotton (McPherson and McPherson 2000), and they have been shown to cause decreased fruit retention, lint staining, lint loss, and seed loss (Greene et al. 2001, Glover et al. 2019). Loss can be further magnified when bacterial boll rot is introduced during probing and feeding activity from stink bugs (Medrano et al. 2015, 2016).

The management of stink bug infestations on cotton currently relies on use of insecticides when action or economic thresholds are exceeding based on visual sampling methods. Methods available include collecting green bolls (2.5 cm in diameter) for internal injury assessment (Toews et al. 2009) and sampling for stink bugs using a drop cloth (Reay-Jones et al. 2009), sweep net (Outward et al. 2008), or beat bucket (Pyke et al. 1980). The sweep net has been found to be more effective sampling nymphs, while the drop cloth was more effective at sampling adults (Reay-Jones et al. 2009), Conversely, the beat bucket has been found to effective at sampling all life stages but efficiencies in its use are affected by plant growth stage. Sampling strategies that displace insects for density estimates are further complicated by the remarkable attachment ability of the stink bug with higher attraction forces (>40) greater than its body weight (Voigt et al. 2019). Sampling time of day and within-plant distribution of stink bugs may also affect the outcomes of monitoring for stink bugs for detection and density estimation. Detection and density estimation of stink bugs require intensive and time-consuming sampling that may be affected by these factors and may introduce bias and variability that negatively affects management.

Biologists have used a wide variety of mark-release-observe techniques to obtain insect observation data related to these issues (Southwood 1978). Rice et al. (2015) demonstrated that marking stink bugs with fluorescent powders and using UV LEDs light sources was a simple, nondestructive and effective technique for detecting, otherwise inaccessible aspects of stink bug distribution, during nighttime in field studies. Cabrera et al. (2016) reported the use of markers of various colors and found no fitness cost or toxicity associated with the marking agent but noted variability in ability to observe different colors.

Information on when (photo-period specific) and where (within-plant distribution) stink bugs are observed on cotton is relevant to these stink bug monitoring and management issues. We used mark-release-observe experiments acquire this information by using a combination of fluorescent marking techniques applied to a representative stink bug and visual blacklight aided observations of the stink bug infesting cotton. The objectives of this study were to assess if stink bugs were observed evenly by plant section (i.e. bottom, middle, and top branches), by fruiting positions and leaf surface, and by concealed or exposed orientation on floral bracts and leaf surfaces across daytime and nighttime observations.

Materials and Methods

In a field study conducted over two years (2016-2017), mark-release-observe experiments were conducted using over five hundred adult green stink bugs to

identify when and where bugs were observed on cotton. Field collected stink bugs were marked or left unmarked with non-toxic fluorescent sharpie markers, released, and monitored in cotton fields at peak bloom. Stink bugs where monitored visually during day and night, aided by a handheld blacklight for nighttime observations. Within-cotton distribution insect observations were categorized by plant section (i.e. bottom, middle, and top branches), by fruiting positions and leaf surface, and by concealed or exposed orientation on floral bracts and leaf surfaces. A simple external marking technique was applied to individual stink bugs which consisted of a quick drying, light weight marker available in several highly visible colors (Walker et al. 1981). The marker was durable, nontoxic, non-water soluble, resistant to peeling and chipping, and easy to apply (Wineriter et al. 1984).

Insect Collection and Pre-infestation Cotton Management

Adult stink bugs were collected on flowering and pod filling stages of soybean in 2016 and 2017. Stink bugs were collected using a modified leaf blower that displaces insects from vegetation and transfers them into an inflatable sock that fits on the opposite end of the blower's fanned nozzle, known as a KISS-sampler (keep it simple sampler) (Beerwinkle et al. 1997). Insects were captured ≈24 h before being released at the base of the cotton plant (Figure 5.1.) in each year. All insects were held individually in plastic portion cups for ≈24 h fasting period and inspected to confirm that only healthy adults (i.e. bugs with all appendages) were used.

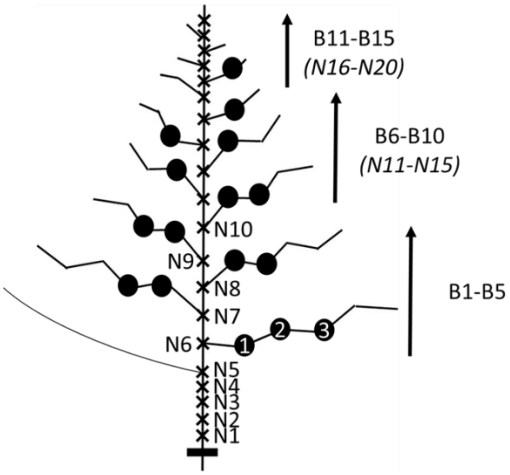

Figure 5.1 Fruiting Branches on Cotton Plant Diagram. Diagram of a cotton plant illustrating fruiting branches present during time of infestation. In 2016 and 2017, stink bugs were released early morning at the base of the plants main stem (N1) during the third week of bloom. Branches were aggregated in groups of 5 sympodial nodes: bottom branches 1-5 (nodes 6-10), middle branches 6-10 (nodes 11-15), and top branches 11-15 (nodes 16-20). Insects were monitored for a period of 3 days and 2 nights, and peak bloom cotton was used, characterized as > 10-12 nodes above first white flower (NAWF).

The mark-release-observe field experiments were conducted in 2016 and 2017 at the Texas A&M AgriLife Research and Extension Center farm in Corpus Christi, TX. Cotton used for the within-plant vertical distribution experiments was selected for uniformity from a ≈1.0 ha field planted to Phytogen 499 WRF (Dow AgroSciences, Indianapolis. IN). Planting occurred in early May on 91-m rows and 96-cm row centers at a rate of ≈77,800 seeds per ha (31,500 seed per acre). Cotton plots were grown without irrigation under dryland growing conditions. Thiamethoxam insecticide (Centric, Syngenta Crop Protection, Greensboro, NC) at labelled rates was used ca. every 10 days to maintain plots pest free before infestation. Thiamethoxam application was discontinued 2 weeks prior to infesting with insects. Other agronomic practices were normal for the region (Morgan 2018).

Field Experimental Design, Marking Technique, and Insect Infestation

Marked stink bugs were released into interior experimental plots nested within a larger contiguous and uniform cotton field to characterize the within-plant vertical distribution of the green stink by plant section (i.e. bottom, middle, and top branches), by fruiting positions and leaf surface, and by concealed or exposed orientation on floral bracts and leaf surfaces. Each experimental plot consisted of 10 consecutive rows (96-cm row centers by 15.3 m in length). Adult green stink bugs were marked with non-toxic neon permeant markers (Sharpie neon fine point permanent markers, Sanford L.P., Oak Brook, IL). The adult bugs were chilled at 3 C in a refrigerator for 2 min in preparation for marking the insects. Individual adult bugs were gently held between the thumb and forefinger, cradled by the middle finger, while the dorsal side was broadly covered with a single marking color of blue or orange neon. To minimize adverse effects to the insects (Wineriter et al. 1984), neon marking ink were only applied to the pronotum extending past the scutellum towards the hemelytra, and including the corium avoiding sensory organs (i.e. antennae, eyes, or wing membranes). The marked and unmarked control bugs were placed in ventilated rectangular plastic and mesh cages (29.2 × 25.4 × 26.6cm bugdorm cages, BioQuip, Rancho Dominguez, California) to air dry.

The candidate marking colors were chosen based on a preliminary darkroom laboratory assay that marked green stink bug cadavers with five neon marker colors (pink, green, yellow, blue, and orange). Marked insects were pinned to live potted cotton plants on leaves and various fruiting structures in a dark room illuminated by a handheld, battery operated portable UVA ultraviolet LED blacklight flashlight (Scorpion Master, 52 LED

395nM, Shawshank Ledz, Gilbert, AZ). Blue and orange had the highest visual reflectance and were therefore selected as the two marking colors along with an unmarked control in the 2016 field experiments. In 2017 field experiments, orange marked bugs along with an unmarked control, were used based on observations in the 2016 experiment (see results section).

In 2016, three experimental plots were infested separately with one of three marking treatments (i.e., blue, orange, and non-marked insects) during the third week of bloom. Third week of bloom was characterized as > ten to twelve nodes above first white flower (NAWF) on the first (mainstem) fruiting position (Kerby et al. 2010). In each experimental plot, 100 stink bugs of an assigned marking treatment were released early morning, before sunrise, at the base of cotton plant main stem approximately at node one (N1) (Figure 5.1). Stink bugs were released ca. every 1.4 m for a total of 10 bugs per row in 10 consecutive rows. A grand total of 300 green stink bugs were used in the experiment (i.e., 100 blue-marked, 100 orange-marked, and 100 non-marked adult stink bugs). In 2017, the same protocol was used in two experimental plots releasing a total of 200 green stink bugs (i.e., 100 orange-marked and 100 non-marked adult stink bugs). Insects were monitored for a period of 3 days and 2 nights in 2016 and 2017.

Photoperiod-specific within-Cotton Distribution Monitoring and Measurements

Insect monitoring and data collection were taken during day and night photoperiods. Daytime measurements were taken during mid-morning when plants were dry from morning humidity (ca. 10:30am). The data collection period was chosen to reflect

a time frame that insect monitoring may occur in a commercial setting. A single observer (JPG) collected all data. The observer walked at a slow pace in-between rows where stink bugs were released. Sampling was initiated at the beginning of each row inspecting all plants within the respective row moving from left to right until all plants within the experimental plot had been observed. The observer walked all experimental plots randomly one after the other over an approximate 45 min time-frame. The observer held the blacklight about 0.5 m from the plants. The sampler visually scanned each individual plant beginning at the terminal moving downward towards the base of the plant to observe the position of stink bugs by plant section (i.e. bottom, middle, and top branches), by fruiting positions and leaf surface, and by concealed or exposed orientation on floral bracts and leaf surfaces. Boll bracts and leaves were gently manipulated to determine the relative concealed or exposed orientation of the stink bug and caution was used to minimally disturb plants. Plants observed with treatment stink bugs were marked with the fluorescent marker to minimize the chances of sampling the same individual multiple times and ensure the independence of stink bug observations. Nighttime measures were taken during the late-evening (ca. 10:30 pm, US central time zone). Measurements were taken during the moon's first quarter (in perigee), on July 12 and July 13 in 2016, and July 30 and July 31 in 2017 (National Weather Service 2016/17).

Within-plant vertical distribution of the green stink bug was assessed by partitioning the plant into bottom, middle, and top sections. Reproductive or sympodial branches were counted in order from bottom to top of the plant, where the bottom branches (B1-5) corresponded to nodes 6-10 beginning with the first reproductive (sympodial)

branch, middle branches (B6-10) corresponded to nodes 11-15, and upper branches (B11-15) corresponding to nodes 16-20 (Figure 5.1). Reproductive branches were consistently found at or above the fifth node above the cotyledonary node. Fruiting and leaf surface distribution data were assessed by recording visual observations of stink bugs on first, second, and third position cotton bolls (N6) (Figure 5.1) or on a leaf surfaces as partitioned among the bottom, middle, and top plant sections. Fruiting positions on reproductive branches were counted in order from the nearest position to the main stem outward (Anderson et al. 2018). Relative concealment and exposure of the stink bugs were assessed by recording observations of the green stink bug in relation to its position on the boll bract and leaf. Cotton bolls are surrounded by three or sometimes four bracts which are the modified leaves at the base of the fruit. Stink bugs observed inside a bract or on a lower leaf surface were recorded as concealed (Figures 5.2A and 5.2D), and stink bugs observed outside a bract (Figures 5.2B and 5.2E) or on an upper leaf surface were recorded as exposed (Figures 5.2C and 5.2F).

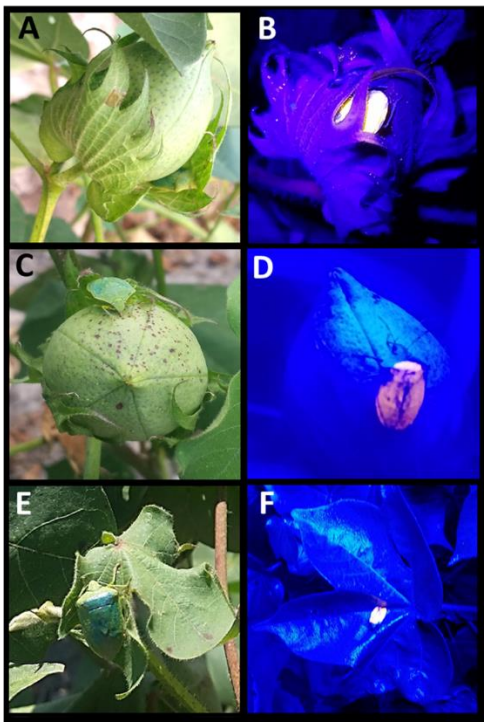

Figure 5.2. Field Measurements. Field measurements taken in 2016 and 2017 photoperiod-specific within-plant distribution experiments. Cotton was infested with marked (fluorescent blue and orange) and non-marked green stink bugs, *Chinavia hilaris* (S.) (Hemiptera: Pentatomidae). Green stink bug observed on a developing first and second poistion cotton boll concealed (inside) the bract (A: day and B: night), green stink bug observed on a first and second position cotton boll exposed (outside) the bract (C: day and D: night), and green stink bug observed on a leaf surface exposed (upper leaf surface) (E: day and F: night).

Data Analysis

The number of stink bugs observed were accumluted and aggregated across three daytime observation periods and two nighttime observation periods, as categorized by plant section, by fruiting and leaf surface distribution, and by relative concealment and exposure on bracts and leaves. A contingeny table analysis was used to test three hypotheses related to these distribution categories separately for daytime and nighttime

observations (Freund and Walpole 1980). The first hypothesis tested for independence of the porportion of stink bugs observed among plant sections (3: top, middle, and bottom) and marking technique (3 in 2016: unmarked, blue-marked, and orange-marked; and 2 in 2017: unmarked and orange-marked). The second hypothesis tested for independence of the porportion of stink bugs observed among plant sections (3) and fruiting and leaf surface distribution (3: first position boll, second position boll, and leaf). The third hypothesis tested for independence of the porportion of stink bugs observed among fruiting position and leaf surface distribution (3) and relative concealment and exposure on bracts and leaves (2: found concealed or exposed on bracts and leaves). The Pearson X^2 statistic was generated and the probability of independence was determined for these three m by n contingency table analyses using the computer program JMP (Lehman et al. 2013).

Results

A total of twelve contingency table analyses were conducted, separated by year, photoperiod, and the three hypotheses. All contingency analyses were significant ($P < 0.05$) for the nighttime observations (6 Pearson Chi-Square tests) and most during the daytime (5 Pearson Chi-Square tests). Cotton can produce bolls beyond the second fruiting position, but very few stink bugs (n < 3 for any observation period) were observed on third or greater position cotton bolls regardless of marking color, photoperiod, and year; therefore, these data were excluded from the analysis.

Within-Plant Distribution of the Green Stink Bug

In 2016 and 2017 daytime field experiments, marked stink bugs were detected at higher rates than non-marked (control) stink bugs (Figures 5.3A and 5.3B) (2016: X^2 = 16.31; df = 4; P = 0.002; total number of stink bugs observed [n] = 56; 2017: X^2 = 1.92; df = 2; P = 0.37; n = 38), respectively. In 2016 field experiments, two florescent colors were assayed (blue and orange). Observations of the number of stink bugs marked with the two marking colors varied but were always higher on the middle and top branches where most of the stink bugs occurred. For experimental efficiency, fluorescent orange was chosen as the single marking color in the 2017 field experiments. In 2016 and 2017 daytime field experiments, more stink bugs were observed in upper portions of the cotton plant (middle and top sections) (Figures 5.3A and 5.3B) during the daytime than in the bottom branches of the plants (Figures 5.1 and 5.3).

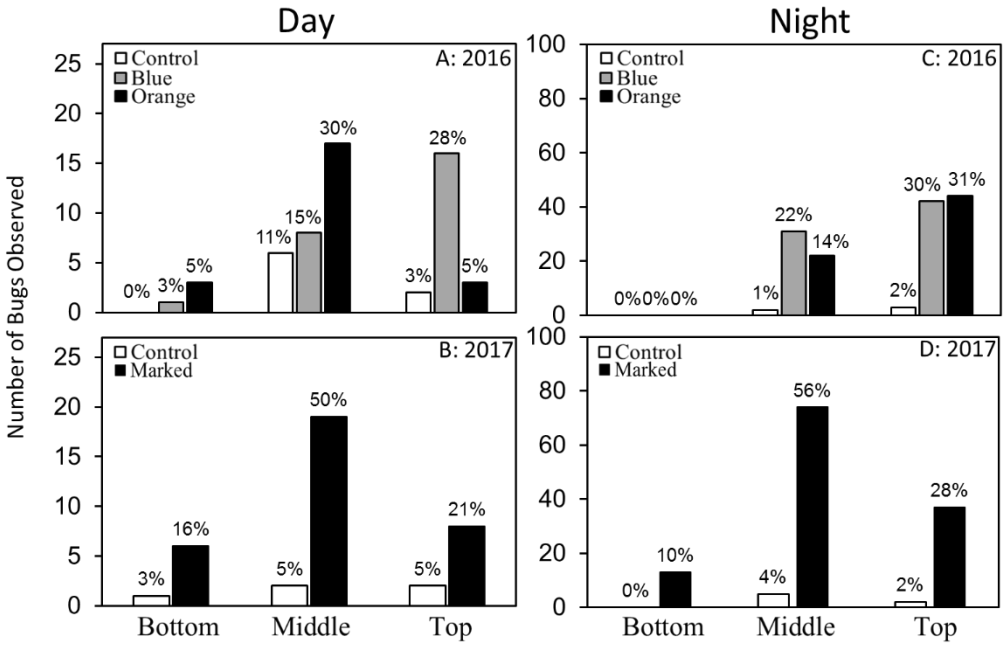

Figure 5.3. Observed Marked and Unmarked Stink Bugs by Branch Sections. Number of marked and unmarked stink bugs observed and percent observed in the daytime (A:2016 and B:2017) and nighttime (C:2016 and D:2017) by branch sections: bottom branches 1-5 (nodes 6-10), middle branches 6-10 (nodes 11-15), and top branches 11-15 (nodes 16-20). See legend for marking treatments used each year.

In 2016 and 2017 nighttime field experiments, marked (fluorescent) stink bugs were detected in greater numbers (n > 140 and n > 130) than non-marked (controls) (n < 7, and n < 3) (Figures 5.3C and 5.3D) (2016: X^2 = 8.65; df = 4; P = 0.013; n = 144; 2017: X^2 = 6.63; df = 2; P = 0.03; n = 169). respectively. Similar to daytime distribution of the green stink bug, marked stink bugs were more frequently observed in upper portions of the cotton plant (middle and top branch sections) (Figures 5.3C and 5.3D) during the nighttime with very few observations (n < 18, both years) on the bottom branches of the plant (Figures 5.1 and 5.3).

Fruiting Position and Leaf Distribution of the Green Stink Bug

In 2016 and 2017 daytime field experiments, significant differences in fruiting site and leaf distribution of marked stink bugs were detected (Figures 5.4A and 5.4B) (2016: X^2 = 10.0; df = 4; P = 0.04; n = 56; 2017: X^2 = 9.94; df = 4; P = 0.04; n = 38) respectively, by plant section (i.e. bottom, middle, and top) (Figure 5.1). In 2016 and 2017 daytime field experiments, marked stink bugs were detected in greater numbers on first position cotton bolls (N6) (Figure 5.1) in the upper portion of the plant (i.e. middle, and top branches) and on second position bolls in the bottom section of the plant (Figures 5.4A and 5.4B). Despite the limited number of observations in 2016 and 2017 daytime field experiments, when marked stink bugs were detected on leaf surfaces bugs were primarily observed in the upper portion of the plant (i.e. middle and top) (Figures 5.4A and 5.4B).

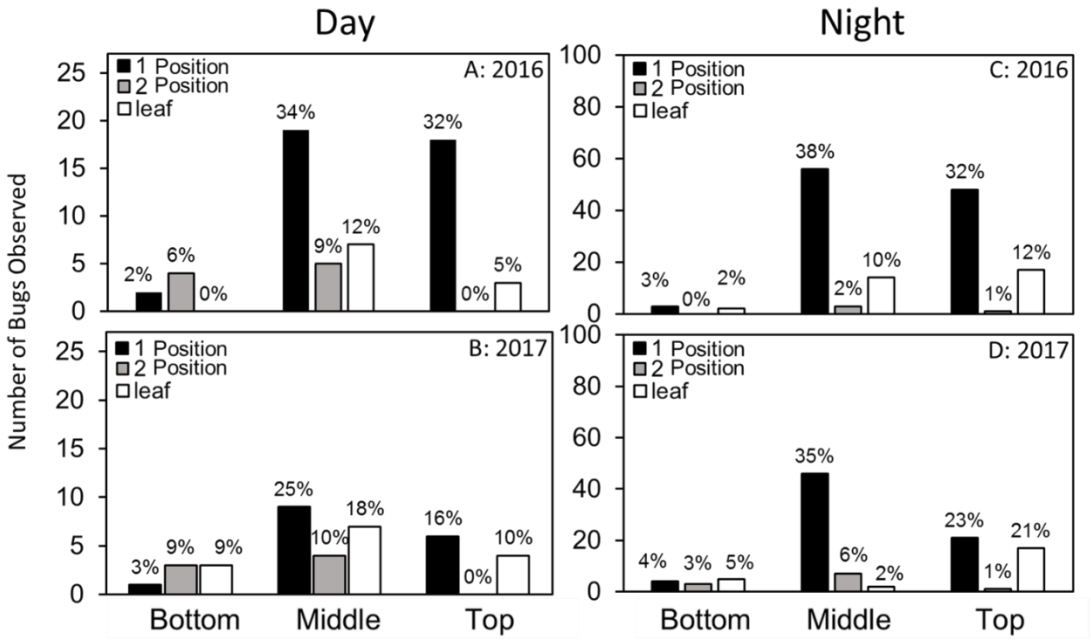

Figure 5.4. Observed Stink Bugs in Daytime and Nighttime by Fruiting Position and Leaf Surfaces. Number of stink bugs observed and percent observed in the daytime (A:2016 and B:2017) and nighttime (C:2016 and D:2017) by fruiting position and leaf surfaces by branch section: bottom branches 1-5 (nodes 6-10), middle branches 6-10 (nodes 11-15), and top branches 11-15 (nodes 16-20). Fruiting positions on reproductive branches were counted in order from the nearest position to the main stem outward. See legend for fruiting position or leaf treatments used each year.

In 2016 and 2017 nighttime field experiments, similar to the fruiting site and leaf distribution observed during the daytime, significant differences in fruiting site and leaf distribution of marked stink bugs were also detected (Figures 5.4C and 5.4D) (2016: X^2 = 14.51; df = 4; P = 0.0005; n = 144; 2017: X^2 = 10.40; df = 4; P = 0.03; n = 131) respectively, by plant section. In 2016 and 2017 nighttime field experiments, marked stink bugs were detected in greater numbers on first position cotton bolls in the upper portion of the plant (i.e. middle, and top branches) and on leaf surfaces in the top section of the plant (Figures 5.4C and 5.4D).

Bract Position Distribution of the Green Stink Bug

In 2016 and 2017 daytime field experiments, significant differences in the stink bug-bract position and leaf surface distribution of marked stink bugs concealment and exposed (Figures 5.2A, 5.2C, and 5.2E) were detected (2016: X^2 = 12.11; df = 2; P = 0.002; n = 56; 2017: X^2 = 7.2; df = 2; P = 0.02; n = 38) respectively, by fruiting position (i.e. first and second position cotton bolls) (Figure 5.1) and leaf surfaces (Figures 5.5A and 5.5B). In 2016 and 2017 daytime field experiments, marked stink bugs were detected at higher rates distributed inside the bract (concealed) (Figure 5.2A) when found on any given fruiting site (Figures 5.5A and 5.5B). Marked stink bugs when detected on leaves were primarily observed on lower leaf surfaces (concealed) (Figures 5.5A and 5.5B).

Conversely, in 2016 and 2017 nighttime field experiments, the opposite trend was observed. Significant differences in the stink bug-bract position and leaf surface distribution of marked stink bugs concealment and exposed (Figures 5.2B, 5.2D, and 5.2F) were detected (2016: X^2 = 11.02; df = 2; P = 0.004; n = 144; 2017: X^2 = 8.23; df = 2; P = 0.01; n = 131) respectively, by fruiting position and leaf surfaces (Figures 5.5C and 5.5D). In 2016 and 2017 nighttime field experiments, marked stink bugs were detected at higher rates distributed outside the bract (exposed) (Figure 5.2D) when found on any given fruiting site (first or second bolls) (Figures 5.5C and 5.5D). Marked stink bugs when detected on leaves were primarily observed on upper leaf surfaces (exposed) (Figures 5.2F, 5.5C, and 5.5D) respectively, in contrast to daytime observations.

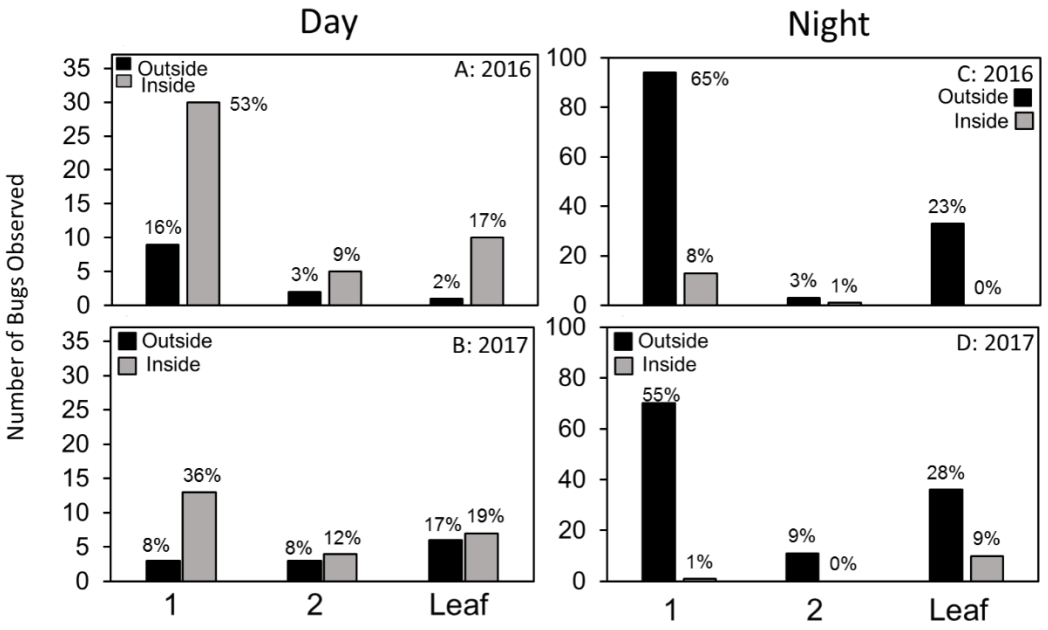

Figure 5.5. Stink Bugs Observed in Daytime and Nighttime Outside or Inside Cotton Boll Bracts. Number of stink bugs observed and percent observed in the daytime (A:2016 and B:2017) and nighttime (C:2016 and D:2017) outside or inside the cotton bolls bract by fruiting position or leaf. Fruiting positions on reproductive branches were counted in order from the nearest position to the main stem outward. See legend for bract position observed each year.

Discussion

This study highlights how differences in photoperiod-specific within-plant distribution of stink bugs may complicates detection of stink bugs using standard sampling methods (Greene et al. 2001, Toews et al. 2009, Reay-Jones et al. 2009). The field experiments spanned two years and were conducted under dryland conditions that are representative of the south Texas cotton-production region using a stink bug representative of this and other production regions (Greene et al 2001).

Observation rates in 2016 and 2017 during the nighttime were on average similar or higher than similar mark-recapture studies (Tillman et al. 2009), demonstrating the effectiveness of the marking technique for stink bugs. The significant differences between daytime and nighttime observations and the differences in within-plant distribution across plant sections, on bolls and leaves, and relative concealment and exposure of the stink bugs may help to explain the difficulties encountered when using current stink bug detection and monitoring methods (Greene et al. 2001, Toews et al. 2009, Reay-Jones et al. 2009).

It can be assumed that marked (fluorescent) stink bugs observed during the nighttime data collection period were experimental insects, not from local populations, and detected after insects went undetected during daytime observations. The larger number of nighttime specific observations of stink bugs in both 2016 and 2017 following the limited number of daytime observations support the conclusion that when an infestation occurs there is a portion of the population that goes undetected during the daytime, which complicates insect density assessment which occurs during the daytime.

Our data suggest candidate sections of the plant to concentrate sampling efforts when monitoring for the green stink bug. Daytime-specific observations indicated stink bugs were distributed 42% and 50% within the upper sections of the plant (i.e. middle) and 35% and 21% top section of the plant in 2016 and 2017 field experiments. Similarly, nighttime-specific observations indicated that bugs were distributed 51% and 56% within the upper sections of the plant (i.e. middle) and 45% and 28% in the top section of the plant during the night in 2016 and 2017 field experiments with very few stink bugs were

observed on the bottom section of the plant. Results further suggest candidate fruiting sites within-plant sections to concentrate sampling efforts when monitoring for the green stink bug. Data support that as the green stink bug moves up the plant from the base it may transition towards the interior of the plant to first position cotton bolls 34% and 25% in the middle section of the plant and in the top section of the plant 32% and 16% in 2016 and 2017, respectively. The same trend was observed during the nighttime data which indicated stink bugs were distributed 38% and 35% on first position cotton bolls in the middle plant section, and 32% and 23% in the top plant section during the night in 2016 and 2017 field experiments, respectively. Furthermore, significant difference in stink bug-bract distributions were detected between day and night observations. Data suggest candidate fruiting sites to survey within-plant sections to maximize sampling efficiency when monitoring for the green stink bug. Daytime-specific observations indicated stink bugs were distributed primarily inside the bract (concealed) (Figure 5.2) when observed on first position fruiting sites 53% and 36%, in 2016 and 2017, respectively. Similarly, stink bugs observed on second position fruiting sites were primarily concealed 9% and 12% inside the bract, observed on lower leaf surfaces 17% and 19% in 2016 and 2017, respectively. In contrast, nighttime observations indicated stink bugs were more exposed, primarily observed outside the bract (> 55%) or on the upper leaf surface (> 23%) (Figure 5.2) in 2016 and 2017, respectively.

The differences in plant section distribution, fruiting and leave position distribution, and relative concealment and exposure during the nighttime in contrast to daytime observation of bugs are important factors to consider when sampling for the green

stink bug. Focusing visual observations within the upper portions of the plant and inspecting boll bracts may improve existing detection and monitoring efforts (Greene et al. 2001, Reay-Jones et al. 2009). Exploration of companion detection techniques may assist in detecting stink bugs in cotton. Xia et al. (2011) reported a fluorescent fingerprint when cotton bolls damaged by stink bugs were exposed to long-wave ultraviolet light. The green bolls emitted a strong blue-green fluorescence in a circular region near the puncture wound. Similarly, stink bugs and their damage have also been detected using an electronic nose (Henderson et al. 2010). Additional research in the applications of fluorescence markers, ultraviolet light, and existing insect detection methods are warranted. Our results here indicate where in the cotton plants, stink bug detection should by focused in isolation or in companion with these developing techniques or the existing technique of opening green bolls to observe visual signs of lint discoloration due to stink bug feeding. In regard to existing stink bug sampling techniques, further field experimentation using the beat bucket, sweep net, and visual observations is warranted to determine if the methods can be further refined using the distribution data from this experiment.

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CHAPTER VI

CONCLUSIONS

The four studies presented in this dissertation culminate in three major conclusions concerning the boll-feeding sucking bug complex present in the coastal bend cotton growing regions of Texas. First, using whole plant caging experiments, plant response to feeding resulted in boll injury in the form of lint deterioration and cotton boll rot at mid and late-bloom stages, and in water limiting and non-water limiting conditions. Although plant injury was apparent across a wide range of conditions, subsequent yield decline attributed to insect feeding was seen primarily under water limiting conditions when plants were infested at mid-bloom. The mid-bloom period of cotton development contained the largest array of susceptible boll ages and represented the more sensitive growth period. Significant yield loss during mid-bloom indicated verde plant bug readily feed on large squares and small bolls which resulted in higher boll abscission under dryland growing conditions and was an important driving factor in yield depression. Second, for these conditions, significant yield—insect density relationships were used to calculate economic injury levels (EILs) for each species. EILs expressed as bugs per plant from lowest to highest were the brown stink bug (0.29 to 0.31 bugs per plant), redbanded stink bug (0.33), verde plant bug (0.49), and green stink bug (0.50). Lastly, the narrowness in the range of economic injury levels for the complex of stink bugs supported the construction and use of a common stink bug EIL of 0.34 bugs per plant. Verde plant bug was less damaging on average and can be considered separately from the stink bugs using a higher EIL of 0.49

bugs per plant (Table 2.3). If a particular stink bug species dominates in an area, the species-specific EIL for a stink bug also may be more appropriate. In a mixed species situation, the common stink bug threshold of 0.34 bugs per plant is a reasonable approach for pest managemen. This study illustrated the importance of the complex of sucking bugs as economic pests of cotton, and highlights how variation of at least one environmental factor, water availability, can affect economic injury levels.

In Chapter 3, using the same species considered in Chapter 2, single boll cage experiments examined the extent that boll age sensitivity to feeding and species differences contributed to differences observed in specific damage and yield when cotton was infested at mid-bloom. Response to feeding resulted in reduced fruit retention, increased boll injury in the form of lint deterioration, and increased cotton boll rot. The contrast of results comparing 0-day, 3-day, 5-day, and 7-day old bolls showed that verde plant bug readily feeds on smaller less mature bolls and was an important contributor to decreased fruit retention. Stink bugs also caused significant injury and boll rot on the older bolls of the study. Secondly, variation in fruit retention, boll injury, boll rot, and yield were primarily associated with species differences rather than between 3-day and 7-day old bolls. The decline in fruit retention and increased boll injury supported the interpretation that main causes of yield decline was poor fruit retention and boll injury that led to cotton boll rot. From a management viewpoint, this has implications on the window of field monitoring that is needed when sampling for these insects.

The variation observed in frequency and magnitude of cotton boll rot in a two-year study suggested a seasonality in the presence of boll rot pathogens or potential differences

in transmission efficiency cross species. Lastly, boll injury was apparent across the species used and subsequent yield decline attributed to insect feeding was detected for all species except the redbanded stink bug. Averaging across boll ages, species-specific yield losses from lowest to highest in grams per boll were the green stink bug (2.9 ± 0.53), verde plant bug (3.5 ± 0.37), brown stink bug (3.8 ± 0.51), and the redbanded stink bug (4.7 ± 0.41) compared to controls (4.6 ± 0.28). In fact, the results of both Chapters 2 and 3 support mid-bloom as the significantly more sensitive blooming period susceptible to boll-feeding sucking bugs. From a management viewpoint, the similarities in fruit retention, boll injury, cotton boll rot and subsequent yield decline observed in these experiments further explains and supports the mid-bloom period of cotton development as containing the largest array of susceptible boll ages, and the more sensitive growth period. Furthermore, the narrow range of yield depression observed in these experiments across stink bug and plant bug infested bolls supported the construction of a common economic injury level for at two species of stink bugs when a mixed species complex is present (see Chapter 2).

Chapter 4 showed the ability for the verde plant bug to harbor, transmit, and retain the cotton seed and boll rotting bacteria, *Serratia marcescens* (CC119-R). This study was the next logical step to expand our work which established decision-making procedures for verde bugs that assumed the presence of boll rot pathogens (see Chapter 2). First, the bacterial infection process did not apparently affect insect vigor based on similar boll injury ratings observed across both *Serratia*-exposed and non-exposed bugs. Cotton bolls with *Serratia*-exposed verde plant bugs had significantly greater presence of *S. marcescens* and symptoms of cotton boll rot than caged without bugs (uninfested controls)

or non-exposed bugs. Secondly, transmission of the disease agent (CC119-R) by the verde plant bug was observed and recovered across all boll ages assayed. Boll rotting bacteria were not detected in locules of a boll that showed no feeding activity and no damaged lint or seed. The magnitude of disease expression was significantly higher on younger 5- and 6-day old bolls than older 7- and 8-day old bolls. Furthermore, pathogen transmission assays detected an ability for the verde plant bug to harbor the disease agent anywhere from 24h up to 96h post-infection and retain the pathogen as it fed on at least two bolls. Lastly, concentrations seemed to remain at or about the same level across the two boll ages considered here.

This study authenticated that verde plant bug injury directly caused by piercing/sucking feeding was distinct from disease infection caused by introduction of *S. marcescens* through the feeding process. Results from these experiments indicated that damage associated with verde plant bug infestations of developing bolls was in large part dependent on whether the insect transmitted the cotton pathogen *S. marcescens*. The use of our disease model provided a method to systematically analyze insect-derived boll injury resulting from verde plant bug feeding/probing alone, and in tandem with boll infection by an insect-vectored pathogen at various stages of fruit development. Appearance of cotton boll rot infection symptoms ranged from reddening of the seed to seed and lint necrosis, and always corresponded with the detection of *S. marcescens* strain CC119-R. Our disease transmission work with the insect vector authenticate presence and persistence of the disease and risk of pathogen transmission by the vector in cotton fields. The nature of bacterial persistence within the insect as seen with the insect's ability to

retain and transmit disease across two bolls may be useful for risk assessment information and for evaluating insect monitoring needs for verde plant bug

Chapter 5 investigated the photoperiod-specific distribution of the green stink bug within the individual cotton plant. Mark-release-observe experiments were conducted to identify when and where bugs were observed on single cotton plants. Stink bugs where monitored visually during day and night, aided by a handheld blacklight for nighttime observations. Within-cotton distribution insect observations were categorized by plant section (i.e. bottom, middle, and top branches), by fruiting positions (first and second position bolls) and leaf surface, and by concealed or exposed orientation on floral bracts and leaf surfaces. First, the green stink bug was primarily (> 80%) distributed in the middle and top branches irrespective of photoperiod, and on bolls in the first position from the main stem (> 62%). Secondly, significant differences in stink bug orientation were detected and varied in daytime and nighttime observations. During the daytime, when observed on fruiting structures stink bugs were primarily observed inside the bract (> 55%), and when detected on leaves on the lower leaf surface (> 54%) concealed. In contrast, nighttime observations indicated stink bugs when observed on fruiting structures were primarily outside the bract (> 66%), and when detected on leaves on an upper leaf surface (> 78%) exposed.

Lastly, our data suggested not only candidate sections within the plant when monitoring for the green stink bug, but also candidate fruiting sites within-plant sections to concentrate sampling efforts. Furthermore, difference in stink bug-bract distributions were detected between day and night observations. The larger number of nighttime

specific observations of stink bugs following the limited number of daytime observations support the conclusion that when an infestation occurs there is a portion of the population that goes undetected during the daytime, which complicates insect density assessment which occurs during the daytime. The differences between daytime and nighttime observations and the differences in within-plant distribution across plant sections, on bolls and leaves, and relative concealment and exposure of the stink bugs are important factors to consider when sampling for the green stink bug and may help to explain the difficulties encountered when using current stink bug detection and monitoring methods. This study highlights how differences in photoperiod-specific within-plant distribution of stink bug complicates detection of stink bugs using standard sampling methods. Focusing visual observations within the upper portions of the plant, on first position cotton bolls, inspecting boll bracts may improve existing detection and monitoring efforts. These results are relevant to refining current stink bug detection and monitoring protocols to improve efficiency and reduce variability.

Together, these studies indicated the overall similarities in plant response from a boll-feeding sucking bug complex. Boll injury from both stink bugs and the verde plant bug took the form of external and internal punctures that upon inspection were visually indistinguishable. Ultimately, many of these probing sites, irrespective of the species and cotton growth stage, became a wart or callous-like mass of proliferated cells. Often, these growths were the initial sites of cotton seed and lint rot collectively referred to as cotton boll rot. Cotton boll rot consistently tracked boll injury: as boll injury increased so did cotton boll rot. Visual symptoms of cotton boll rot vectored by stink bugs and verde plant

bug at harvest ranged from moderate to severely matted and discolored lint, rotted seed and or lint, groups of desiccated locules, and or whole bolls that abscised from the plant.

Bolls exhibiting both boll injury and cotton boll rot occurred during mid and late-bloom, but only caused significant economic yield depression during mid-bloom. The experiments in this dissertation did not look at disease progression or symptomology at various times post-infection. However, it should be noted that a limited exploration of green boll samples from both the whole plant and single cage experiments, irrespective of species that contained cotton boll rot, consistently had symptoms ranging from reddening of the seed to seed and lint necrosis. Bolls experienced cotton boll rot during both mid and late-bloom, but only caused a significant economic yield depression during mid-bloom. However, the degree to which the different species can successfully or competently vector cotton boll rot varied across species and years during field experimentation. One interpretation is that boll response to some species may be less severe in terms of cotton boll rot frequency or the species is a less competent vector.

Fruit retention was a major driving factor in significant yield decline when cotton was infested with verde plant bug. Decreased fruit retention was observed at similar or greater rates for the verde plant bug when compared to stink bugs, especially on younger less mature fruit. The verde plant bug is considerably smaller compared to the stink bugs considered in these experiments. The studies in this dissertation, comparing members of the boll-feeding sucking bug complex, reemphasized that size is not a reliable indicator of an insect's injury and disease potential as observed with the verde plant bug. Lastly, time inefficiencies and variability in current detection efforts may be reduced by focusing our

efforts in the upper portions of the cotton plant on first position bolls, physically inspecting the floral bract, and looking at lower leaf surfaces during routine periods of insect monitoring.

While I explored important yield-insect density relationships to generate drought-sensitive economic injury levels, future work to define non-water limited economic injury levels for areas that receive above average annual precipitation or affected by El Niño weather patterns may prove useful for finer management. Second, the lack of boll rot observed in bolls caged with the redbanded stink bug and the differences in frequency of occurrence of cotton boll rot for other species observed may be associated with differences in habitat reservoirs for the disease. Species such as the redbanded stink bug were collected in soybean, while the other species were collected in sorghum. Differences in the efficiency of cotton boll rot transmission were observed to some degree across species. Detailed cross-species disease transmission and vector competency studies may help explain what drives the variation detected across species in this study. From a management viewpoint, further research should be conducted to determine if insects are more likely to be infected with a boll rotting pathogen when populations transition from either overwintering weedy hosts or alternative row crop hosts into cotton acting as habitat reservoirs for the disease. Furthermore, pathogen transmission assays detected an ability for the verde plant bug to harbor the disease agent anywhere from 24h up to 96h post-infection and retain the pathogen as it fed on at least two bolls. Future work should be done do determine how long verde plant bug remains infective and able to successfully transmit disease.

Lastly, additional research in the applications of fluorescence markers, ultraviolet light, and existing insect detection methods are warranted. Our results indicate where in the cotton plants stink bug detection should be focused, possibly in companion with future cotton boll rot field-detection techniques or the existing technique of opening green bolls to observe visual signs of lint discoloration due to stink bug feeding. In regard to existing stink bug sampling techniques, further field experimentation using the beat bucket, sweep net, and visual observations is warranted to determine if these methods can be further refined using the distribution data from the mark-release-observe experiments.