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Ecology of Megacopta cribraria (Hemiptera: Plataspidae) and Implications for Management on Soybean

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ECOLOGY OF *Megacopta cribraria* (HEMIPTERA: PLATASPIDAE) AND
IMPLICATIONS FOR MANAGEMENT ON SOYBEAN

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Entomology

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by
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ABSTRACT

Megacopta cribraria (Fabricius) (Hemiptera: Plataspidae) has spread through thirteen states and the District of Columbia since accidental introduction into the United States in 2009. The potential impacts to soybean production necessitated research into the ecology and management of the plataspid in its new invasive range. A two year study in South Carolina using sweep-net and beat-cloth sampling generated sampling plans for *M. cribraria* population estimates and sequential sampling plans for pest management decision making. At all adult and nymph densities fewer sweep net samples were required for population estimations compared with the number of beat cloth samples. Sequential sampling reduced the sample size required to reach a management decision for both sampling methods compared with a fixed sampling plan. The sweep net method was more cost reliable for population estimation at low densities of both life stages; the beat cloth became more cost reliable as populations increased. The beat cloth method was more cost reliable than sweep-net sampling across all densities and life stages for pest management practices. White cross-vane traps as a sweep-net sampling alternative on soybean were evaluated. Adults were first collected in traps before the sweep net. Adults collected from trap and sweep-net sampling decreased from the field edge towards the field interior. There was a positive association between the two sampling methods, but only 36% of weekly, by location analyses were significantly correlated. Collected females were rated based on egg development. Female populations of all reproductive maturities dispersed into soybean but proportions varied with sampling method. Kudzu, *Pueraria montana* Loureiro (Merrill) variety lobata (Willdenow) plays an important role in the bivoltine life cycle of *M.*

cribraria so sweep-net sampling was employed to monitor seasonal activity over three time periods, in two kudzu patches in South Carolina. Adults colonized kudzu in early April and were present until October. The majority of females were intermediate or fully reproductive in early spring, whereas non-reproductive females dominated as the season progressed. Soybean stem-feeding behavior was conducted on adult females using electropenetrography and was the first time a member of the Plataspidae has been recorded using this technique. Waveforms were described and correlated with stylet insertion by staining for salivary sheaths. Adult females performed a stereotypical set of feeding behaviors and stylet sheaths terminated in vascular tissue during ingestion. Scanning electron microscopy images of *M. cribraria* mouthpart structures were produced and described. In 2014, nematodes were reported infecting *M. cribraria* adults for the first time, and were subsequently found in nymphs of *M. cribraria, Euschistus servus* (Say)*, Chinavia hilaris* (Say) and adults of *Euschistus* spp. in 2015. Infection in less mobile nymphs suggests the insects were parasitized directly in the soybean field from which they were collected. Morphological and molecular analyses confirmed the nematode belongs to the family Mermithidae, genus *Agamermis*. Overall, the research presented in this dissertation presents insights into *M. cribraria* biology and ecology and provides information to facilitate sampling practices for improved reliability of *M. cribraria* estimates for research and pest management purposes. Entomoparasitic nematode infection in hemipteran pests emphasizes the need to acknowledge covert, under-studied natural enemies which may contribute to population regulation.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

In 2009, *Megacopta cribraria* (Fabricius) (Hemiptera: Plataspidae) sightings in northeastern Georgia, United States established the first time the species had been reported outside of central and southeastern Asia, Australia, and the collective islands of New Caledonia (Eger et al. 2010). An urban and soybean *Glycine max* (L.) Merrill, pest, the plataspid rapidly spread through southeastern US soybean production areas and is now present in thirteen states and the District of Columbia (Ruberson et al. 2013, Gardner 2016). Prior to 2009, the most recent *M. cribraria* research (as the conspecific *M. punctatissima*) was performed almost exclusively in Japan and focused on mating biology (Hosokawa and Suzuki 2001, Himuro et al. 2006) and the Plataspidae-unique endosymbiotic system (Fukatsu and Hosokawa 2002, Hosokawa et al. 2005, Hosokawa et al. 2006, Hosokawa et al. 2007, 2008). The limited management and ecological information, especially related to soybean, is partly attributed to infrequent pest status in the Old World. The potential threat to a $34.5 billion US soybean industry (USDA-NASS 2016) and the danger of an export trade embargo to Central America (Gardner et al. 2013a) necessitated applied research for developing sustainable management strategies. A substantial amount of information regarding *M. cribraria* management, biology, and ecology in the US is now available, but research is still needed to fill in vital research gaps. Here, I review and summarize the *M. cribraria* literature to date from both native and invasive ranges, before and after introduction into the US.

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Taxonomic Nomenclature

First described from Indian specimens as *Cimex cribraria* by Fabricius in 1798, later taxonomic classifications included three other genera: *Tetyra, Thyreocoris,* and *Coptosoma* before being placed in the genus *Megacopta* (from Greek *megas*, great, from Latin *coptero*, covering) in 1977 (Eger et al. 2010). The specific epithet "*cribraria*" is the Latin word for sieve, which aptly refers to the dark stippling observed on the adult scutellum, resembling holes in a sieve. Inclusion of *M. cribraria* in a genetic study to analyze pentatomomorphan relationships strongly suggests that the species has a close association with the families Scutelleridae and Pentatomidae (Li et al. 2005). The family Plataspidae, falls into the monophyletic superfamily Pentatomoidea, which in turn belongs to the second biggest infraorder, Pentatomomorpha, in the order Hemiptera.

Megacopta punctatissima (L. *punctum*, spotted, *-issimus*, very), described by Montandon in 1896, is slightly dissimilar in color and size from *M. cribraria* (Hosokawa et al. 2014) but both "species" can interbreed and produce fecund offspring (Hosokawa et al. 2007). These observations, complemented with genetic studies, provide evidence that the two "species" are conspecific, although phenotypically diverse. While the Japanese literature still refers to two different species (Hosokawa et al. 2007, Hosokawa et al. 2014), this review will acknowledge conspecificity by including research regarding both *M. cribraria* and *M. punctatissima*.

Numerous region-specific common names have been attributed to *M. cribraria*, including the bean plataspid (Ahmad and Moizuddin 1975, Zhang et al. 2012), negro bug (Borah and Sarma 2009a), kudzu bug (Ruberson et al. 2013), and lablab bug (Sujithra et

al. 2008). The species is also frequently described as a stink bug (Fukatsu and Hosokawa 2002, Wu et al. 2006, Jenkins and Eaton 2011), although this most often refers to insects in the family Pentatomidae. The Entomological Society of America accepted the request for the frequently used common name of "kudzu bug" (referring to the host plant on which the insect was first discovered in the US) to be included in the authorized Common Names of Insects and Related Organisms list in 2014 (Entomological Society of America 2016). This name will be used hereinafter, when appropriate.

Invasion History and Distribution

In October 2009, specimens and photos of an unknown insect were received by the University of Georgia (UGA) Homeowner Insect & Weed Diagnostics Laboratory from several counties in northeast Georgia (Suiter et al. 2010). Adults had been observed on homes and vehicles, and both adults and nymphs had been observed on the vines of kudzu, *Pueraria montana* Loureiro (Merrill) variety *lobata* (Willdenow) Maesen & S. Almeida. Following location visits and collections, researchers identified *M. cribraria* using morphological characters (Eger et al. 2010). Subsequent genetic analysis directed at a fragment of the cytochrome oxidase subunit I gene by researchers at UGA confirmed the morphological identification (Jenkins et al. 2010).

Reported *M. cribraria* distributions, prior to arrival in the US, included two countries in Oceania (Australia and New Caledonia) and thirteen countries in Asia ranging between Pakistan and Japan, and Indonesia and China. In these areas, the insect was known to function primarily as a legume feeder (Eger et al. 2010). Although the pathway

responsible for US introduction remains undetermined, mitochondrial haplotype analysis has indicated the most southwesterly of Japan's four main islands as the invasion origin (Hosokawa et al. 2014). This constitutes only the second report of a species from the family Plataspidae to be observed west of the prime meridian (Froeschner 1984). The first, *Coptosoma xanthogramma* (White), the black stink bug, was discovered on one of Hawaii's northern islands in 1965 (Beardsley and Fluker 1967). Thought to originate from the Philippines, this plataspid established quickly and decimated the indigenous legume, *Sesbania tomentosa* (Hook & Arn), within a decade (Howarth 1985).

In response to discovery and apparent rapid spread, Pest Alerts were created by members of the Department of Entomology at UGA, the Georgia Cooperative Extension Service, the Georgia Department of Agriculture, and the United States Department of Agriculture (USDA-PPQ). Designed to notify and educate Extension, research, and pest management personnel, the alerts also requested feedback on the ever-increasing county distribution (Suiter et al. 2010). Within a year, the initial distribution of nine counties in northeast Georgia had expanded 14-fold in area to over 80 counties in three additional states (Alabama, North Carolina, and South Carolina). In June 2010, the plataspid was first found infesting soybean in Georgia and South Carolina. In light of this, appeals were made for timely research efforts regarding host-plant screening, surveillance, damage quantification, and management strategies (Eger et al. 2010, Suiter et al. 2010). A *Megacopta* Working Group comprised of researchers at Clemson University, North Carolina State University (NCSU), UGA, Emory University, and Dow AgroSciences was founded. This multi-state collaboration established a website to provide up to date

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information for homeowners, soybean growers, and first detectors, which included a continually-updated, editable distribution map (Gardner 2016).

Promptly after invasion, Zhu et al. (2012) used ecological niche modeling to predict the potential distribution of *M. cribraria* in the US using climate, topography, and habitat, but without host plant consideration. In addition to Georgia, North Carolina, and South Carolina (which at the time were already infested), Alabama, Tennessee, Mississippi, Louisiana, Virginia, Florida, and Louisiana were predicted areas of high suitability. Habitat suitability reduced westwards and northwards from the documented "index case" in Georgia. Indeed, the actual spread followed the predicted pattern; seven states were infested by 2012, and by 2015, over 610 counties, municipalities, and parishes in thirteen states (Alabama, Arkansas, Delaware, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, South Carolina, Tennessee, and Virginia), and the District of Colombia, were reported to have encountered *M. cribraria*.

Rapid spread has been attributed to a strong flying ability coupled with weather fronts, air currents, host plant availability, and phoretic vehicular movement along major highways in the Southeast (Ruberson et al 2013, Takano and Takusu 2016). This natural dispersal, combined with long distance dispersal or "stratified dispersal," is characteristic of many invasive pests (Liebhold and Tobin 2008). Microsatellite primers have been developed to examine *M. cribraria* gene flow and population structure as expansion continues (Jenkins et al. 2013). The ongoing project will provide explanations for how a small initial population with little genetic variation succeeded in establishing in a novel

environment, similar to examples in other invasive pests (Tsutsui et al. 2000, Grapputo et al. 2005).

Biology and Ecology

External Morphology. Adult *M. cribraria*, glabrous and box-like in shape, have two-segmented tarsi and an enlarged, punctate scutellum covering the fore wings and abdomen. These characteristics separate Plataspidae from other families of US-inhabiting Pentatomoidea (Eger et al. 2010). The US acanthosomatids, of which there are five species in two genera, also possess two-segmented tarsi but have a small, triangular scutellum (Rider 2012). Aiyar (1913) reported that non-entomologists erroneously identify *M. cribraria* as a beetle, presumably due to the hypertrophied scutellum that resembles a coleopteran elytra. Over 100 years later, misidentification is still apparent (Roney 2011, Wallace 2014). Adults are morphologically dimorphic externally, with the slightly larger females possessing a pale venter around y-shaped terminal sternites and triangular genital segments while males have rounded, darker terminal sternites with a circular genital segment (Eger et al. 2010, Zhang et al. 2012). Physical dimensions and color vary within and between both native and invasive ranges, but adults generally range from 3.5-6.0 mm in length and are light brown to olive green in color (Eger et al. 2010, Brown et al. 2014).

Aiyar (1913) superficially described and illustrated eggs and nymphs collected from southern India (as *C. cribraria*). A comprehensive set of descriptions, illustrations, and accompanying tables of measurements were published by Moizuddin and Ahmad (1975) from collections made in Karachi, Pakistan (as *C. cribrarium*) and also by

Thippeswamy and Rajagopal (2005b) from collections made in Bangalore, India (as *C. cribraria*). Specimens collected in the US have also been described alongside reproduced photographs in Eger et al. (2010) and Zhang et al. (2012). In brief, nymphs are oval in shape, the red first instar changes color to brown, then yellowish-green, and later to the highly mobile, greenish-brown fifth instar with well-defined wing pads, that extend to the third abdominal segment. Instars increase in length from approximately 0.8-4.45 mm, and dorsum abdominal hair density increases through instar development.

    Internal Morphology. Researchers from Pakistan (Ahmad and Moizuddin 1974, 1975) focused on describing and illustrating *M. cribraria* (as *C. cribrarium*) from multiple specimens, after deeming sample sizes used in general heteropteran morphological studies unsuitable (Miyamoto 1957, Pendergrast 1957, Miyamoto 1961). They anticipated larger sample sizes would enhance evaluation of character importance in determining Plataspidae classification at the superfamily and family level, as well as provide insight in understanding internal organ function.

    The adult scent apparatus consists of a flat, leaf-like gland and an orange sac-like pouch, reaching from the metasternum to the fifth abdominal segment. The gland ejects secretions though connecting ducts to the pouch. Ostioles, located between the mesothoracic and metathoracic coxal cavities, permit ejection via elongated tubes or vestibules. Little is known about chemical composition of the secretion, but it is thought to closely resemble that of pentatomids with a corresponding defense and aggregation function (Aldrich 1988).

Fukatsu and Hosokawa (2002) used scanning electron microcopy to build on the alimentary canal observations and illustrations of Ahmad and Moizuddin (1974). Although different annotations were employed, general descriptions correlated between the two studies. Females possess specific sections identified as the swollen crypt bearing midgut and brownish enlarged midgut end (Fukatsu and Hosokawa 2002) or midgut five (Ahmad and Moizuddin 1974). These sections are responsible for the production of the symbiont capsule components (see Symbionts).

Males possess seven testicular follicles in each testes, and females likewise possess seven ovarioles in each ovary [reported in Miyamoto (1957) as the Japanese *C. punctissimum,* in Thippeswamy and Rajagopal (2005b) as the Indian *C. cribraria,* and from US *M. cribraria* specimens collected in Alabama by Golec and Hu (2015)]. Insects classified in the lower Pentatomoidea, are understood to possess twelve chromosomes ($2n = 10 + X + Y$), which has indeed been reported in the karyotype of *M. cribraria* (Satapathy and Patnaik, 1991).

Symbionts. The first phytophagous, hemipteran gut symbiont molecular study was performed on *M. punctatissima* (Fukatsu and Hosokawa 2002), and the species is generally regarded as a model organism for gut symbiont phylogeny research. Females harbor capsules within the posterior midgut, and these comprise three components; (1) envelope, (2) matrix, and (3) a ɣ-proteobacterium symbiont, *Candidatus* Ishikawaella capsulata [hereinafter *Ishikawaella*, the epithet referring to Hajime Ishikawa who pioneered insect symbiosis studies (Fukatsu and Hosokawa 2002, Hosokawa et al. 2005)]. Envelope and matrix components are produced by and stored in female-specific specialized sections of

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the midgut (Hosokawa et al. 2005). Simultaneous with egg lay, females deposit capsules beneath the egg mass. Maternal control of the symbiont ensures one capsule is produced for every 3-4 eggs. Emerged nymphs immediately probe the egg mass and ingest *Ishikawaella* (a consistent titer of $2x10^7$ symbionts), ensuring a critical stationary phase for 1-2 days (Hosokawa et al. 2007, 2008). Phylogenetic analyses indicate that the insect and symbiont have undergone strict cospeciation (Hosokawa et al. 2006), and nymphs deprived of *Ishikawaella* will develop slowly and die (Fukatsu and Hosokawa 2002). Egg-capsule manipulations have determined that one capsule is sufficient for normal development of six nymphs, hence females produce a symbiont excess. Pest status is possibly connected to *Ishikawaella* genotype (Hosokawa et al. 2007). Experimentally exchanging the symbiont between *M. punctatissima* (considered a pest) and *M. cribraria* (considered a non-pest) reverses *M. punctatissima* success on soybean, due to high nymph mortality or reduced egg hatch (Hosokawa et al. 2007). Reciprocally, *M. cribraria,* when provided with the *M. punctatissima* symbiont, exhibits pest characteristics. Sequence similarity with the aphid (Hemiptera: Aphididae) endosymbiont, *Buchnera aphidicola,* and tsetse fly (Diptera: Glossinidae) endosymbiont, *Wigglesworthia glossinidia* Akoy*,* suggests that *Ishikawaella* may also manufacture essential amino acids for host use (Nikoh et al. 2011).

Symbiont work has not been restricted to Asia; gene sequences amplified from individual *M. cribraria* collected in Georgia and South Carolina revealed two vertically transmitted endosymbionts: *Ishikawaella* and *Wolbachia* (one strain) (Jenkins et al. 2010). The US *Ishikawaella* had the same pest-conferring genotype 16S sequence as the *Ishikawaella* isolated from the pest *M. punctatissima*, as well as 99% sequence similarity

to the groEL sequence from *M. cribraria* and *M. punctatissima* collected in Japan (Jenkins and Eaton 2011, Nikoh et al. 2011).

Development and Mating Behavior. Old World researchers have described the life history of *M. cribraria* on various host plants (Ahmad and Moizuddin 1977, Tayutivutikul and Yano 1990, Srinivasaperumal et al. 1992, Thippeswamy and Rajagopal 2005b, Shi et al. 2014). Similarly, researchers in the US have evaluated development, reproduction, and survival on proposed host plants in both laboratory and field conditions (Zhang et al. 2012, Medal et al. 2013, Blount et al. 2015, Golec et al. 2015) (see Host Plants). Although host plant, environmental factors, and research methods affect life history parameters, general observations can be made. Females lay up to 49 eggs in two parallel rows with the operculum facing outwards, commonly on plant tips at the top of the stratum. Egg hatch time is positively correlated with temperature and occurs in the morning hours (Thippeswamy and Rajagopal 2005b). Oviposition to hatch duration of 3.5-4.5 days has been reported at 27°C (Srinivasaperumal et al. 1992), whereas a decrease by 10°C can extend incubation time by up to 14 days (Shi et al. 2014). Eggs survive between 17°C and 33°C, but nymphs are unable to molt to adults at 33°C. Nymphs pass through five stadia, with the average developmental time increasing as temperature decreases. Fifth instars spend a longer time developing compared to other instars. RNAi experiments performed on *M. punctatissima* show that *laccase2* gene is essential for normal adult cuticular maturation and pigmentation after the final nymph molt (Futahashi et al. 2011). Male and female longevity varies widely; Srinivasaperumal et al. (1992) report only three days on hummingbird tree, *Sesbania grandiflora* (L.) Poiret, firecracker, *Crossandra*

infundibuliformis (L.) Nees (as *C. undulaefolia*) and cotton, *Gossypium hirsutum L.* at 27°C, whereas Shi et al. (2014) report 76 days at 25°C and 44 days at 29°C on soybean. It is likely that longevity also differs between generations.

Adult *M. cribraria* aggregate on host plants to mate (Hibino and Ito 1983, Hibino 1985). Aggregation sizes average around four, but clumps of up to 25 individuals have been observed (Hibino and Ito 1983). Aggregations consist of mating pairs plus males not in copula (awaiting female arrival) (Hibino 1985). Males play an active role in aggregations through initiation, maintenance, and courtship, whereas females attend aggregations solely to mate (Hibino 1985). Females favor copulation with larger males (Himuro et al. 2006) that aggregate together (Hibino and Ito 1983), hence female choice is the selective force for male gregariousness. Copulations can be over 24 hours in the laboratory and up to 10 hours in the field (Hosokawa and Suzuki 2001). Matings are most likely to occur in the early afternoon until early morning the next day, which may relate to predator (see Natural Enemies) abundance or optimal time of subsequent oviposition (Hosokawa and Suzuki 2001, Thippeswamy and Rajagopal 2005b). Four hours is required for sperm transfer to female spermatheca; hence remaining copulation time is considered a form of mate guarding (Hosokawa and Suzuki 2001).

Hibino (1986) compiled a courtship ethogram beginning with male and female antennae contact upon encounter. The male moves to the female abdomen tip, maintaining antennae contact with the female body. After turning around, the male, with its genital tube projected, thrusts towards the female abdomen. Copulation ensues if the female remains

stationary. Retreat or raising of the abdomen results in male pursuit and continued courtship.

Host Preferences and Feeding Behavior. Legumes (order Fabales, family Fabaceae) are the primary host plants of *M. cribraria.* Eger et al. (2010) reference 20 host legume species in Asia, including lablab bean, *Lablab purpureus* var. *lignosus* Medikus, kudzu, soybean, and pigeon pea, *Cajanus cajan* (L.) Millsp. Several non-leguminous hosts have also been recorded (Eger et al. 2010). In Japan, adult *M. punctatissima* ($n=2$) have been observed feeding and damaging *Rhus chinensis* (Mill) (Chinese sumac) galls, produced by the aphid *Schlectendalia chinensis* (Bell) (Yamazaki and Suguira 2005). The presence of *M. punctatissima* on nearby leguminous plants suggest a facultative use of galls as a food source.

Eight months following discovery near and on kudzu patches, *M. cribraria* adults were found on soybean plants (Suiter et al. 2010). Observation, field tests, and greenhouse evaluations have confirmed that soybean and kudzu are the primary developmental hosts of *M. cribraria* in the US (Zhang et al. 2012, Gardner et al. 2013a, Medal et al. 2013, Seiter et al. 2014a, Blount et al. 2015, Golec et al. 2015). Observations during the initial spread of the insect indicated that nine of 33 plant species recorded (17 legumes, 16 non- legumes) were present in the Asia host record list (Eger et al. 2010, Gardner et al. 2013a). Eggs together with nymphs and adults were observed only on kudzu and soybean, suggesting that some records were random alighting, resting, or mating aggregation events rather than true developmental hosts. Lovejoy and Johnson (2014) analyzed adult midgut DNA by amplifying and sequencing the chloroplast *trn*L gene. Leguminous plant DNA (kudzu,

soybean, white sweet clover, *Melilotus alba* Medik, lablab, bushclover, *Lespedeza* sp. Michx., and peanut *Arachis hypogaea* L.) was detected. Furthermore, each insect midgut contained more than one plant species including non-legumes such as walnut, *Juglans* sp. L., sweet gum, *Liquidambar styraciflua* L., tomato, *Solanum lycopersicum* L., lettuce, *Lactuca sativa* L., sorghum, *Sorghum bicolor* (L.) Moench, and pine, *Pinus* spp. L. A non-leguminous host range correlates with plant records compiled by Gardner et al. (2013a). It is important to understand the complexity of *M. cribraria* host plant interactions. Bridge-hosts may be significant for development and essential for population increase before dispersal, as observed in pentatomid pests (Panizzi 1997). Furthermore, broad herbivory may have contributed to the rapid coverage witnessed in the southeastern US and suggests that a wider range of US geographic distribution and establishment is possible.

Host-preference tests have also been performed on edible beans and alternative legume species with economic importance in the US and Central America. These experiments have included field trials (Blount et al. 2015) and laboratory choice/no-choice tests using adults collected directly from the field (Zhang et al. 2012, Medal et al. 2013, Seiter et al. 2014a, Blount et al. 2015), or overwintered adults deprived of kudzu (Golec et al. 2015). There is general consensus that in addition to soybean and kudzu, edamame and pigeon pea are developmental hosts. Reports regarding host status for black-eye pea *Vigna unguiculata unguiculata* (L.) Walp., lima bean, *Phaseolus lunatus* L., and pinto bean, *P. vulgaris* L. are contradictory, and reevaluations are needed to determine the degree to which each function as a developmental and/or feeding host.

Observational reports indicate that *M. cribraria* feed on plant sap, mainly towards the top of the stem of their host plant, or at the base of the petiole (Hibino and Ito 1983, Tayutivutikul and Yano 1990, Thippeswamy and Rajagopal 2005b, Thippeswamy and Rajagopal 2005a, Kikuchi and Kobayashi 2010, Seiter et al. 2013a). Although Wu et al. (2006) and Thippeswamy and Rajagopal (2005b) describe adults and nymphs feeding on bean pods, there is no evidence of direct damage to the seed (Seiter et al. 2013a). A reliable tool for understanding and monitoring insect feeding behaviors and mechanisms is electropenetrography (EPG), where an insect and plant are wired into an electrical circuit (McLean and Kinsey 1964). Stylet insertion into the plant closes the circuit and activities, such as ingestion, are converted into records of voltage change called waveforms which can be visualized. The voltage change is obtained from the resistance change produced from the varying conductivity of the saliva, phloem sap, and water that circulate through the mouthpart apparatus. Acquired waveforms can be identified, described, quantified, and statistically analyzed. Three studies have been performed on soybean-feeding pentatomid species: *Nezara viridula* (L.) (Cooke 2014), *Edessa meditabunda* (F.) (Lucini and Panizzi 2016)*,* and *Piezodorus guildinii* (Westwood) (Lucini et al. 2016). The data collected include time until first probe, probe duration, and when correlated with histological studies, have identified target ingestion tissue. Results from EPG studies permit feeding behavior comparisons with different life stages, host and non-host plants, or insecticide applications, and enables examination of temporal feeding behaviors (Cook and Neal 1999, Joost and Riley 2005). Integration with pest life history and ecological studies have implications for

pest management strategies such as insecticide selection, sampling, and resistant plant cultivar development.

Seasonal Activity and Population Dynamics. Seasonal activity and population dynamics have been characterized on kudzu in south Japan (Tayutivutikul and Yano 1990) and on lablab bean (as *Dolychos lablab*) in Karachi, Pakistan (Ahmad and Moizuddin 1977). One to three generations per year are reported in southeast China, with adults emerging from overwintering in middle to late March, and peak adult densities occurring in May and June, and again in September and October (Wu et al. 2006). Information from other Old World locations can be assembled from sampling dates reported in other *M. cribraria* research publications, such as those examining parasitoids. For example, eggs were collected by Hirose et al. (1997) from soybean through July and August in Fukuoka, Japan with a July egg peak correlating with adult colonization of soybean fields (Takagi and Murakami 1997).

Population dynamics in the US, where two generations are produced per year, have been documented in kudzu (Zhang et al. 2012) and soybean (Seiter et al. 2013b). During spring, overwintering adults become active and disperse to kudzu and early-planted soybean (Del Pozo-Valdivia and Reisig 2013). Aggregation, feeding, mating [15% of females are mated pre-overwinter (Golec and Hu 2015)], and egg laying result in the first new generation of the year (Zhang et al. 2012). First generation adults typically peak in June. Adult flying ability enables the first complete generation to invade soybean plants or kudzu patches and begin a second ovipositional period (Ruberson et al. 2013). The second peak occurs in August, and these adults form the overwintering generation. Reported

overwintering sites include beneath the loose bark of pine (Lahiri et al. 2015), pecan, *Carya illinoinensis* (Wangenh.) K. Koch (Golec and Hu 2015), and sweet gum, beneath leaf litter (Lahiri et al. 2015), and inside residences (Ruberson et al. 2013).

Overcrowding has been proposed as a dispersal cue from kudzu to soybean (Zhang et al. 2012). Dispersal ability and flying behavior are, however, poorly understood. Dispersal capacity assessments of overwintered, first generation, and second generation populations relative to reproductive status and different hosts are required. Flight mill studies are currently underway (Attisano et al. 2015, Moore, unpub. data), as are also mark-recapture studies in North Carolina, South Carolina and Georgia (Knight, Reay-Jones, Reisig and Toews, unpub. data). Adult *M. cribraria* movements were apparently captured as 'backscatter echoes' on weather radars, in Virginia (Melnikov et al. 2015) and the technique shows promise for future research (Chapman et al. 2003).

Natural Enemies. In two Chinese coastal provinces, egg parasitism by an encyrtid, *Ooencyrtus* sp., was observed in soybean fields (Zhang et al. 2003, Wu et al. 2006) and played an important role in reducing *M. cribraria* populations. Similarly, Hirose et al. (1997) reported *O. nezare* Ishii parasitizing between 82-100% of egg masses (= 65-69% of total eggs) sampled in July and August in the Kyushu region of Japan. Likewise, *Paratelenomus saccharalis* (Dodd) (Hymenoptera: Platygastridae) parasitizes egg masses in China [described as *Asolus minor,* now synonymous with *P. saccharalis*] (Johnson 1996, Wu et al. 2006), Japan [described as *P. minor*] (Hirose et al. 1997, Takagi and Murakami 1997), and India (Srinivasaperumal et al. 1992). In Japan, Takagi and Murakami (1997) collected non-parasitized egg masses from kudzu and exposed them to mated female *P.*

saccharalis. Wasps emerged in 11 days (30ºC) to 25 days (20ºC), with the highest parasitism rate (88%) occurring at 27.5ºC.

Adult *P. saccharalis* have emerged from egg masses collected in Georgia, Alabama, Mississippi (Gardner et al. 2013b), and Florida (Medal et al. 2015). Specific to parasitizing the family Plataspidae, this is the first time the species has been reported in the Western Hemisphere. It is presumed that parasitized eggs were introduced on shipments of plants, nursery stock, or food products.

Generalist, natural enemies have also been reported in the US. *Phasia robertsonii* (Townsend) (Diptera: Tachinidae) was recovered from a single adult in Georgia (Ruberson et al. 2013) and Golec et al. (2013) reported *Strongygaster triangulifera* (Loew) (Diptera: Tachinidae) in 5% of the 214 adults dissected from April soybean collections. Ruberson et al. (2013) observed the predatory pentatomid *Euthyrhynchus floridus* (L.) attacking adults in the field, and a range of geocorids, reduviids, coccinellids, and a chrysopid larvae attacking nymphs in the laboratory. A formal study by Greenstone et al. (2014) analyzed predator gut contents. Two geocorids, *Geocoris punctipes* (Say) and *G. uliginosus* (Say)*, one anthocorid, *Orius insidiosus* (Say)*, one pentatomid, *Podisus maculiventris* (Say)*, one coccinellid, *Hippodamia convergens* Guerin-Meneville*, one reduviid, *Zelus renardii (*Kolenati)*, two oxyopid spiders, *Oxyopes salticus* (Hentz) and *Peucetia viridans* (Hentz), and one formicid, *Soleopsis invicta* Buren screened positive for *M. cribraria* DNA.

Borah and Dutta (2002) report the entomopathogenic fungus *Beauveria bassiana* (Balsamo) as a natural biocontrol agent of *M. cribraria* in India, affecting up to 32% of all nymphs and adults encountered in pigeon pea fields in 1997. In laboratory studies, adult

and nymph mortality was 5-7 times greater at concentrations between $1x10^3$ and $1x10^7$ *B. bassiana* spores/ml, compared to a distilled water control (Borah and Sarma 2009b). Infection of *M. cribraria* (in patches of kudzu and soybean fields) with *B. bassiana* (Balsamo) Vuillemin clade A was first described in South Carolina in 2012 (Seiter et al. 2014b) and has been observed in subsequent years in the state (pers. obs.).

The microsporidium, *Nosema* Nägeli, was isolated and characterized from *M. cribraria* adults collected in Guangzhou, China (Xing et al. 2014). Spores were fed to silkworm, *Bombyx mori* L., larvae and resulted in systemic infection of the midgut, silk glands, gonads, muscle, fat body, and Malpighian tubules, and could be subsequently transmitted transovarially. *Nosema* presumably maintains infection in *M. cribraria* populations due to nymph oral acquisition of the symbiont capsule. It is unknown how *Nosema* affects *M. cribraria.*

Pest Status and Economic Importance

Agricultural pest. Soybean yield losses in China (Wang et al. 1996, Xing et al. 2006), Japan (Takagi and Murakami 1997, Hosokawa et al. 2007, Kikuchi and Kobayashi 2010), and India (Thippeswamy and Rajagopal 2005a) have been reported. Similarly, *M. cribraria* has been described as a pest of other legumes: hummingbird tree (Srinivasaperumal et al. 1992), lablab bean (Ahmad and Moizuddin 1977, Thippeswamy and Rajagopal 1998, 2005b, Sujithra et al. 2008), and pigeon pea (Borah and Sarma 2009a), as well as the non-legume firecracker (Srinivasaperumal et al. 1992). Pest status is also

classified as negligible in some areas of Asia (Eger et al. 2010), most likely a result of effective natural enemies.

Studies in 2010 and 2011 in Georgia and South Carolina in 19 untreated soybean fields showed an average yield loss of 18% from *M. cribraria* damage, ranging from 0-56% (Ruberson et al. 2012). Cage trials, confining adults to soybean plots, effectively evaluated *M. cribraria* density-yield loss relationships. At the highest initial density of 25 adults per reproductive soybean plant, densities reached 184 nymphs and adults per plant over the growing season, culminating in a 60% yield reduction. The seed per pod and seed weight reduction was attributed to feeding induced stress during seed fill (R5) and full seed (R6) (Seiter et al. 2013a).

There are currently over 24 pest species from five orders with the potential to damage soybean in the southern US (Musser et al. 2015). An annual survey, representing 11 million soybean acres from seven US states (Mississippi, Tennessee, Arkansas, Alabama, Louisiana, North Carolina, and Virginia), first reported soybean yield losses from *M. cribraria* in 2011 (Musser et al. 2012). In Alabama alone, the kudzu bug contributed 10% of the total soybean losses and control costs due to insect pests in 2013. From 2011 to 2014, over $6.5 million of soybean yield losses plus insecticide treatment costs have been attributed to *M. cribraria* in the southern US, which are an underestimation of costs, as Georgia and South Carolina did not factor into these surveys (Musser et al. 2012, 2013, 2014, 2015).

In 1970 kudzu was formally classed as a weed and in 1997 was placed on the Federal Obnoxious Weed List in the US (Forseth and Innis 2004). Indeed, the extensive

root system makes it impossible to permanently eliminate it, and the ability to outcompete native species results in forest productivity losses estimated at over $100 million per year (Forseth and Innis 2004). Current methods for managing kudzu include frequent, expensive, and time consuming defoliation and herbicide usage (Forseth and Innis 2004). Hence, a common misconception amongst the American public proposes that *M. cribraria* was deliberately imported as a means of kudzu control (pers. obs.). Interestingly, a cooperative program funded by the USDA Forest Service initiated to survey kudzu natural enemies in China named *M. cribraria* as one of the 116 phytophagous insects found on the legume (Sun et al. 2006). Although *M. cribraria* feeding damage can reduce kudzu leaf, stem, and total biomass by 32.3%, 32.6%, and 32.5%, respectively (Zhang et al. 2012), any benefit provided is enormously outweighed by the potential damage to the valuable US soybean crop (USDA-NASS 2016) and is a clear counter argument for "intentional introduction" statements.

Urban and Medical Pest. Flying swarms and the physical appearance of aggregations on white surfaces such as gutters, windows, and home exterior siding, combined with the strong, foul odor emitted on disturbance, attracted the attention of home and business owners, thus classifying *M. cribraria* as an urban pest (Suiter et al. 2010). Bracketing *M. cribraria* into a "pests of medical importance/interest" category can also be advocated as secretions create burning sensations and leave itchy red welts on skin (Ruberson et al. 2013). In Hong Kong, the closely related *M. centrosignatum* (Yang) can cause inflammation and infection of the eyelid, presumably by physical irritation of the mass in the eye, in conjunction with emitted secretions (Wong and Mak 2012). Medically

diagnosed as "preseptal and orbital cellulitis," this condition has the potential to be life or sight threatening if not treated in a timely and judicious manner (Watts 2016).

Management

Monitoring and Sampling. Pest monitoring and sampling is a critical component of integrated pest management (IPM) programs and provides a basis for targeting tactics. As with other soybean hemipteran pests (Rudd and Jensen 1977, Kogan and Pitre 1980), sweep-net sampling has been the primary tool to sample both adult and nymph stages for research. The technique is also recommended for monitoring and scouting in soybean to classify whether the insect population is above or below the published threshold, before intervention decisions are made (Seiter et al. 2013c). Beat cloths are also used to sample overground soybean hemipteran pests (Kogan and Pitre 1980), and the technique has also been utilized in *M. cribraria* research (Greenstone et al. 2014). Based on a design by Ulyshen and Hanula (2007), white cross-vane traps have been suggested as another method for sampling and monitoring the mobile adult. Adults are attracted to a white cross-vane, and a soapy water-filled bucket below the cross-vane captures flying insects as they encounter the vanes and fall. Zhang et al. (2012) used cross-vane traps to monitor adult activity from May 2010 to May 2011 in kudzu patches and reported trap catches of up to 2,000 adults per trap in kudzu during a single week, while traps operated by Horn and Hanula (2011) collected an average of 60 adults per trap per week over seven weeks in kudzu.

Chemical Control. Insecticide applications are the only *M. cribraria* management options currently available for soybean growers. Insecticide efficacy evaluations were conducted in 2010-12 in South Carolina and North Carolina with organophosphate, pyrethroid, neonicotinoid, carbamate, chitin synthesis inhibitor, oxadiazine, and molting hormone agonist chemical classes (Seiter et al. 2015a). Across all trials, pyrethroid and carbamate classes provided the highest immediate percentage control of *M. cribraria* (2-6 days after application), of which bifenthrin was the most effective (both biologically and economically) active ingredient. Current management strategies recommend applying insecticide whenever sweep-net sampling or visual inspections of the canopy reach one nymph per sweep (Seiter et al. 2013c). A study conducted in Georgia, North Carolina, and South Carolina suggests that a single, well timed application at soybean growth stage R3 or R4 is sufficient to prevent yield losses, as well as the recommended application when nymphs reached one per sweep (Seiter et al. 2015b). Additional, subsequent applications (for example, if the recommended threshold was reached for a second time) had no yield advantage and merely increased economic costs (Seiter et al. 2015b). Further evaluations with higher thresholds for subsequent applications have been recommended. An additional study investigating the differences in susceptibility of soybean to *M. cribraria* damage based on plant phenology highlighted the period two to six weeks after R2 (corresponding to pod and seed development) as the most critical for *M. cribraria* management to prevent soybean yield reductions (Seiter et al. 2016).

Knowledge of insect spatial distributions can aid in the formation of comprehensive sampling plans and IPM strategy development (Taylor 1984, Binns and Nyrop 1992, Reay-

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Jones et al. 2009). Adult and nymph *M. cribraria* have an aggregated distribution in India on pigeon pea, based on Taylor's power law and Iwao's patchiness regression (Borah et al. 2002), and on soybean in Japan, based on the goodness of fit to the Poisson distribution (Kono 1990). Seiter et al. (2013b) reported *M. cribraria* aggregations in soybean in South Carolina, with greater abundance along field edges. This suggests that border applications of insecticide have potential as a management strategy, but further research and robust data are needed.

In the US, the outer perimeters of infested houses have been sprayed with pyrethroids by pest management professionals to control *M. cribraria* as a nuisance pest (Suiter et al. 2010). This has occurred when insect populations have disrupted normal outside leisure activities for homeowners. Evaluations of professionally used insecticides on different exterior building materials such as brick and plywood indicated that pyrethroids, neconicotinoids, pyrethroid-neonicotinoid mixes, and oxadiazines were effective at controlling *M. cribraria* (Seiter et al. 2013d).

Cultural Control. Cultural practices such as planting date manipulation can affect beneficial and pest insect densities (Buschman et al. 1984, Pedigo and Zeiss 1996, Gore et al. 2006). Early-planted soybeans in the southeastern US are more prone to *M. cribraria* infestation, presumably because they can harbor both generations (Blount et al. 2016, Del Pozo-Valdivia et al. 2016). Planting from the middle of May onwards may avoid egg-laying by dispersing overwintered adults. Prudence has been recommended regarding planting date manipulation as soybean yield potential is not only impacted by insect densities but also by other interlinking environmental factors.

Biological Control. Classical biological control, the importation and introduction of a natural enemy to a new location where they do not occur naturally, is a viable approach for invasive insect pest management (Kimberling 2004). Success is based on extensive biological and ecological studies of natural enemy and pest with host specificity regarded as the main criterion for species selection (Mills 2005). Parasitic effectiveness, searching, reproductive and dispersal ability, and adult longevity contribute to effective reduction of pest numbers by an imported organism (Caltagirone and Huffaker 1980, Mills 2005).

USDA scientists at the Agricultural Research Service facility in Stoneville, Mississippi identified *P. saccharalis* as an ideal candidate for importation. Positive results following quarantine trials, screening, and evaluations with US native species were eagerly anticipated (Gardner et al. 2013a, Ruberson et al. 2013), but as the research was near completion, *P. saccharalis* was discovered in Georgia (see Natural Enemies), and thus the importation process was halted. This scenario is not unique. Three years after accidental introduction of *C. xanthogramma* into Hawaii, a non-native egg parasitoid *Trissolcus* sp. (Hymenoptera: Platygastridae) was reared from eggs collected in the field and had likely entered the country with its host (Davis 1978).

Identified *M. cribraria* predators disperse between cotton and soybean fields suggesting that adjacent soybean-cotton habitats may augment *M. cribraria* predation (see Natural Enemies) and contribute to management as part of a conservation biological control approach (Greenstone et al. 2014).

Host Plant Resistance. Host plant resistance (HPR) is recognized as a cost-effective, environmentally friendly IPM strategy to manage phytophagous pests (Bansal et

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al. 2013). This strategy encompasses non-preference, tolerance, and antibiosis (Painter 1958) and has successfully managed soybean pests in the past, such as the potato leaf hopper, *Empoasca fabae* Paoli (Bansal et al. 2013). Recent studies in North Carolina (Fritz et al. 2016) and Georgia (Bray et al. 2016) have added to the two previous Chinese-language publications (Xing et al. 2006, Xing et al. 2008) that evaluated soybean HPR to *M. cribraria.* Fritz et al. (2016) tested soybean genotypes that included various maturity groups, pubescence type, leaf shape and size, drought tolerance, seed protein content, and pest resistance. Narrow-leaf, small seeded soybean genotypes (N7103 and Vance) were the most resistant in the field. The development of a 'kudzu bug resistance index' incorporating damage ratings and adult and nymph numbers to screen lines may prove useful for future evaluations of soybean genotypes (Bray et al. 2016).

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CHAPTER II

DEVELOPING SAMPLING PLANS FOR THE INVASIVE *Megacopta*

cribraria (HEMIPTERA: PLATASPIDAE) IN SOYBEAN

The invasive *Megacopta cribraria* (Fabricius) (Hemiptera: Plataspidae), a species endemic to Asia, was first discovered in northern Georgia, United States, in 2009 (Suiter et al. 2010). Initially regarded as a nuisance pest due to high numbers around homes, emissions of unpleasant odors, and the tendency to discolor the skin when handled, it was later elevated to an agricultural pest due to its infestation of soybean, *Glycine max* (L.) Merr. (Suiter et al. 2010, Ruberson et al. 2013). A variety of legumes are reported as host plants from the native range of *M. cribraria* (Eger et al. 2010), but soybean and kudzu, *Pueraria montana* (Lour). Merr. variety *lobata* (Willd.), are the most important hosts in the United States (Suiter et al. 2010, Zhang et al. 2012). By the end of 2013, the range of *M. cribraria* had expanded to include most of the southeastern states (Gardner et al. 2013).

Studies in Georgia and South Carolina in 2010 and 2011 showed an average yield loss of 18% from *M. cribraria* damage (Ruberson et al. 2013). Furthermore, cage trials by Seiter et al. (2013b) showed that soybean yield was reduced by up to 59.6% at high densities of *M. cribraria.* Corresponding reductions in seeds per pod and seed weight were attributed to stress induced by *M. cribraria* feeding primarily on the stem during the seed fill stage (R5) and full seed development stage (R6) (Fehr and Caviness 1977). Existing broad-spectrum insecticides already utilized in soybean are effective against *M. cribraria* in the United States (Ruberson et al. 2013) and in their native range (Zhixing et al. 1996).

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Current management strategies recommend application of insecticide at a threshold of one nymph per sweep or when nymphs can be observed from 10 visual canopy inspections per field (Greene et al. 2012).

Having a method to assess insect population density through sampling plans is a vital component of integrated pest management (IPM). Due to the relatively new incursion of *M. cribraria* into the United States, no comprehensive sampling plans have been developed. Behavioral aspects of the target species, its developmental stage, cost of sampling equipment, sampling speed, and host-plant stage often affect the sampling method chosen as the most appropriate and effective (Turnipseed and Kogan 1976, Shepard 1980). Additionally, knowledge of insect spatial distributions can aid in the formation of comprehensive sampling plans (Taylor 1984, Binns and Nyrop 1992).

Sampling of insects is used in research to determine population estimates that are as close to the population mean as possible and in pest management to classify an insect population as above or below an economic threshold to enable decisions to be made on applying an intervention (Binns and Nyrop 1992, Hutchison 1994, Wilson 1994). Sequential sampling plans can reduce insecticide applications and overall sampling costs in many crop systems compared with fixed sampling plans (Shepard 1980, Hoffmann et al. 1991, Binns and Nyrop 1992, Schexnayder et al. 2001). The objectives of this study were to (1) determine sample sizes for population estimates for adults and immatures of *M. cribraria*, (2) develop and validate sequential sampling plans for the immature stages of *M. cribraria*, and (3) determine the relative-cost efficiency of the methods used to sample adults and nymphs in South Carolina soybean fields.

Materials and Methods

Field Sampling. Populations of *M. cribraria* were monitored in four soybean fields in 2012 (two at the Clemson University Edisto Research and Education Center [Edisto REC], Blackville, SC, and two at the Clemson University Pee Dee REC [Pee Dee REC], Florence, SC) and four soybean fields in 2013 (one at the Edisto REC and three at the Pee Dee REC). Field size ranged from 2.3 to 6.5 ha with an average of 4.6 ha in both years. In each field, a uniform sampling grid consisted of one sampling location (marked with a 1.8-m fiberglass flag) every 0.12 ha, with flags separated by 35 m. Number of sampling locations within fields ranged from 25-40 (2012) and 24-58 (2013) due to field shape and size.

At each sampling location, two sampling methods were compared. The first method required a 0.91-m by 0.91-m white beat cloth to sample 1.83 m of row at Edisto REC and 3.7 m of row at Pee Dee REC. After placing the beat cloth under the soybean plant canopy between two rows, the plants were vigorously shaken on both sides of the beat cloth to dislodge any *M. cribraria*. Adults and nymphs were counted in the field and recorded. Adults and nymphs counted in Florence were halved before analysis to standardize densities between Edisto REC and Pee Dee REC. The second method consisted of swinging a 38-cm-diameter sweep net across two soybean rows at each sampling location. Each sample consisted of two subsamples of 10 sweeps which were summed before analyses. Samples were collected in plastic bags (30-cm x 50-cm) and frozen (-20°C) in the laboratory before adults and nymphs were counted. Sampling was conducted biweekly in Blackville, SC, in 2013 and weekly in all other fields in both years from late vegetative

stage (V8) until late reproductive stages (R6) when the plants became too mature to sweep.

Number of sampling dates ranged from 7-14 in 2012 and from 6-12 in 2013. On 16 August

2013, in Blackville, SC, insecticide applications were made with *gamma*-cyhalothrin

(Declare®; 0.017kg [AI] / ha). Sampling resumed the following week. No insecticide was

applied in any other field. In selected fields in both 2012 and 2013, a stop watch was used

to record time taken to carry out the required tasks of each sampling method, including

sampling time in the field, walking time between sample points, and processing time for

samples in the laboratory.

Taylor's Power Law. Means and variances of densities of adults and nymphs were

calculated for each field at each date. The spatial patterns of *M. cribraria* adults and

nymphs were described according to Taylor's power law (Taylor 1961, Taylor 1984) which

relates mean density to variance (equation 1):

$$s^2 = ax^b \qquad\qquad [1]$$

where s^2 is the sample variance, x is the sample mean, and a and b are Taylor's coefficients.

Calculations were performed in SigmaPlot (SigmaPlot 2006) using nonlinear regression

rather than simple linear regression after log transformations, which can overestimate

variance at low insect densities (Wilson 1994). Outlying observations, as determined by

Cook's distance, on the regression estimates were deleted and the regression was repeated

(Mendenhall and Sincich 2011) to obtain Taylor's coefficients a and b. Cook's distance

*v*alues that exceeded the 50th percentile of an *F* distribution with $k + 1$ and $n - (k + 1)$

degrees of freedom were deleted, with k = number of terms in regression and n = number

of observations. Coefficient b was compared with a value of 1 using a *t*-test [t = (slope −

1) / (SE of the slope)], with df = n - 2 and P = 0.05 (Zar 1999). Variance to mean ratios were calculated from raw data points.

Optimum Sample Size for Population Estimates. Calculated means and variances were also used to determine the sample size (n) required for population estimation within a given level of reliability. The equation of Karandinos (1976) as modified by Wilson and Room (1983) was used:

$$n = t^2_{\alpha/2} D_x^{-2} s^2 x^{-2} \tag{2}$$

where $t_{\alpha/2}$ is the standard normal variate for a two-tailed confidence interval, D_x is the ratio of half the chosen confidence interval to the mean, and x is the mean population density. The substitution of the sample variance (s^2) from equation 1 in equation 2 gives:

$$n = t^2_{\alpha/2} D_x^{-2} ax^{b-2} \tag{3}$$

Equation 3 was used to obtain estimates with 90% confidence (α = 0.1) within 10, 20, or 30% (D_x = 0.1, 0.2 and 0.3) of the mean for optimum sample sizes using the sweep net and beat cloth.

Sequential Sampling. Equation 4, which incorporates both type I (α) and type II (β) errors, was used to develop sequential sampling plans for nymphs for the beat-cloth and sweep-net methods (Wilson 1994):

$$n = t^2_{\alpha-\beta} |x - T|^{-2} ax^b \tag{4}$$

where n is sample size, t is the standard normal variate for a one tailed confidence interval, α is the type I error rate of declaring that an insect population is above a threshold when it is not, β is the type II error rate of declaring that an insect population is below a threshold when it is not, x is the population density, T is the economic threshold expressed as the

mean density per sample unit, and *a* and *b* are Taylor's coefficients. The current economic threshold used for the sweep-net method is one nymph per sweep (Greene et al. 2012). A linear regression relating beat-cloth data to sweep-net data collected from the same sampling location was used to determine the equivalent economic threshold for the beat-cloth method corresponding to this threshold (PROC REG, (SAS Institute 2010). Sequential plans were developed using error rates $\alpha = \beta = 0.1$ and $\alpha = \beta = 0.2$.

Cost-Reliability. A ratio of costs for beat-cloth and sweep-net methods was used to compare the two sampling methods (Wilson 1994). This identified the better method for estimation of insect population (equation 5):

$$C_{bc} / C_{sn} = n_{bc} (\theta_{bc} + \varphi_{bc}) / [n_{sn} (\theta_{sn} + \varphi_{sn})] \qquad [5]$$

where C_{bc} and C_{sn} are the cost per sample in time for a given level of reliability for the beat-cloth and sweep-net methods, n_{bc} and n_{sn} are the number of sampling units required either for 1) a population estimate with a given level of reliability (D_x) or 2) classification of an insect population above or below the economic threshold for a given level of reliability for the corresponding sampling method, θ_{bc} and θ_{sn} are the times required to carry out the required sampling (both sampling and enumerating) for the beat cloth and sweep net, respectively, and φ_{bc} and φ_{sn} are the times required to move between each sampling location. It was assumed that the time required to move between sampling location was the same for either method, hence $\varphi_{b} = \varphi_{sn}$. Equation 5 calculates the relative cost reliability of the sweep-net method compared with the beat-cloth method based on sample units and time required to carry out the corresponding method. Using equation 3 to

replace *n* in equation 5 and including the previously calculated parameters of the linear regression equation determines equation 6:

$$\frac{C_{bc}}{C_{sn}} = a_{bc}(A + Bx)^{(b_{bc}-2)}(\theta_{bc} + \varphi_{bc})/[(a_{sn}x^{(b_{sn}-2)})(\theta_{sn} + \varphi_{sn})] \quad [6]$$

where C_{bc} / C_{sn} is the relative cost reliability of the sweep-net compared with the beat-cloth method, a_{bc} and b_{bc} are Taylor's coefficients for the beat-cloth method, a_{sn} and b_{sn} are Taylor's coefficients for the sweep-net method, A and B are the intercept and slope of the linear regression relating beat-cloth to sweep-net counts, and *x* is mean population density expressed in number of *M. cribraria* per sample unit (20 sweeps). When *n* is replaced in equation 5 with equation 4 for pest management sampling and with addition of the linear regression equation (Table 2.2), we obtain:

$$\frac{C'_{bc}}{C'_{sn}} = B^{-2}a_{bc}(A + Bx)^{b_{bc}}(\theta_{bc} + \varphi_{bc})/(a_{sn}x^{b_{sn}})(\theta_{sn} + \varphi_{sn}) \quad [7]$$

where C'_{bc} / C'_{sn} is the relative cost-reliability of the beat-cloth method compared with the sweep-net method. Equations 6 and 7 were used to determine cost reliability of both methods for commercial pest management and population estimation, respectively.

Comparison of Fixed and Sequential Samplings Plans. To compare sequential sampling plans with a fixed sampling plan we used *n* = 10 as a fixed sample size, where each sample equates to 20 sweeps. Two additional fields were sampled creating a total of 20 new field-date combinations (14 in Florence in 2012 and six in Blackville in 2013). The number of *M. cribraria* nymphs caught after each sample unit was recorded. Numbers were compared with the corresponding sequential sampling plans developed for beat-cloth and sweep-net methods. Both error rates were used ($\alpha = \beta = 0.1$ and $\alpha = \beta = 0.2$) and sample size and decision as to whether an intervention was required, not needed, or whether

sampling must continue were recorded. Mean sample sizes for each sequential plan at each error rate ($\alpha = \beta = 0.1$ and $\alpha = \beta = 0.2$) were calculated. Fixed and sequential sampling plans were compared using a one-sample t-test (SAS Institute 2010). Percentage of sample size reduction for sequential sampling plans with respect to the fixed sample plan was calculated.

Results

Across both years of this study, 23,760 adults and 18,048 nymphs were collected with sweep-net sampling. Densities of adults and nymphs per 20 sweeps averaged 7.2 ± 0.5 (SEM) (range 0-698) and 4.5 ± 0.4 (range 0-276), respectively. Across both years of study, 15,828 adults and 12,564 nymphs were counted with beat-cloth sampling. Densities of adults and nymphs per 1.83-m of row averaged 5.5 ± 0.3 (range 0-435) and 4.5 ± 0.3 (range 0-325), respectively. The corresponding economic threshold for the beat cloth was calculated as 24.7 nymphs per 1.83m of row (Table 2.1)

Taylor's coefficients and variance/mean ratios are reported for each life stage (Table 2.2). The *b* parameter of Taylor's power law was significantly different from 1 ($P < 0.05$) for nymphs with either method (Table 2.2), indicating aggregated distributions. The number of sample units required to arrive at an estimate within a given reliability for different population estimates was expressed as the number of *M. cribraria* per 20 sweeps (sweep net) and 1.83 m of row (beat cloth) for both nymphs and adults (Fig. 2.1). To obtain a population estimate within 10, 20, or 30% ($D_x = 0.1$, 0.2 or 0.3) of the mean for both the sweep-net and beat-cloth methods, the number of sample units was higher at lower

densities compared with higher densities. At levels of reliability of 10% ($D_x = 0.1$) for the sweep-net method, the number of samples exceeded 5,000 in the case of adults (Fig. 2.1A), in comparison with ~600 samples for nymphs (Fig. 2.1B). At levels of 30% reliability ($D_x = 0.3$), the number of samples required was considerably smaller; at economic threshold densities of one nymph per sweep (20 nymphs per 20 sweeps), optimum sample sizes were 184, 48, and 22 within 10, 20, and 30% of the mean, respectively (Fig. 2.1B). Similar results were found for population estimation using the beat-cloth method. At the corresponding threshold for nymphs with the beat-cloth method (24.7 nymphs per 1.83 m of row), optimum sample sizes were 239, 62, and 29 within 10, 20, and 30% of the mean, respectively (Fig.2.1D). Population estimates required fewer sweep-net samples compared with beat-cloth samples at all densities for both adults and nymphs. For example, at a density of 20 adults per 20 sweeps, 24 sweep net sample units would be required for population estimation whereas 32 beat cloth sample units would be required at the same density of adults ($D_x = 0.3$).

Sequential sampling plans are shown for *M. cribraria* nymphs (Fig. 2.2). When the cumulative number of nymphs for the corresponding sample unit number falls in the "Stop sampling, intervention not needed" area, a management intervention is not required, and the sampling can stop at that time. When the cumulative number of nymphs falls in the "Stop sampling, intervention needed" area, a management intervention is needed, and sampling can stop at this point. Finally, when the cumulative number of nymphs falls in the "Continue sampling" area, sampling must continue until a decision on whether to intervene can be made. A minimum sample size of four was selected as the minimum

number of sample units required as suggested by Shepard (1980) for sampling other arthropods in soybean.

The average times needed to sample and count both adults and nymphs of *M. cribraria* for the sweep-net and beat-cloth methods were 159.0 ± 8.0 s ($n = 202$) and 25.9 ± 0.7 s ($n = 332$), respectively. Time for the sweep-net method was significantly greater than that for the beat-cloth method ($t = 16.58$; df $= 204$; $P < 0.001$). The time taken to walk between sampling locations averaged 28.7 ± 0.1 s ($n = 70$). The sweep-net method was more cost-reliable at values above one (Fig. 2.3). The beat-cloth method was more cost-reliable at values below one. For population estimation at low densities, the sweep net was the more cost-reliable of the two methods for both life stages. For adults, the beat-cloth method became more reliable at densities of 2.8 adults per 20 sweeps. For nymphs, the beat-cloth method became more reliable at densities of 2 nymphs per 20 sweeps. For pest management, the beat-cloth method was most reliable across all population densities for both life stages.

Sample size required to reach a decision using the 0.1 error rate with sweep net sequential sampling plans was significantly smaller compared with the fixed sample plan of 10 sample units ($t = 85.73$; df $= 19$; $P < 0.001$). The average number of 20 sweep units required with the sequential sampling method was 4.1 ± 0.1 compared with the 10 sample size requirement for the fixed plan method. This illustrates a 59% reduction in sample size using the sequential plan for sweep nets (Table 2.3). The sample size required using an error rate of 0.2 with the sweep-net sequential sampling plan for all the 20 field-date combinations was four (Table 2.3). This was also the case when using the beat-cloth

sampling plan ($\alpha = \beta = 0.1$ and $\alpha = \beta = 0.2$). This illustrates a 60% reduction in sample size using the sequential sampling plan compared with using the fixed sampling plan.

Discussion

Most insect species show clumped spatial distributions over a wide range of population densities (Taylor et al. 1978). Parameter *b* in Taylor's power law represents the aggregation index of a particular life stage of an insect in a given environment, with $b = 1$ highlighting a random (Poisson) distribution, $b < 1$ representing a uniform or overdispersed distribution, and $b > 1$ denoting a clumped or aggregated distribution (Taylor 1961, Wilson 1985). Our calculated *b* parameter (Table 2.2) suggests that nymphs of *M. cribraria* have an aggregated distribution with both the sweep-net (as shown also in Seiter et al. [2013b]) and beat-cloth methods ($P < 0.05$). Although the *b* parameter was not significantly different from one for adults, the variance/mean ratio suggests there is a degree of aggregation associated with this life stage at certain densities (Table 2.2). Wilson (1985) highlights that a small value of *b* (< 1) and a high value of *a* (>1), can describe a variance/mean ratio by which distribution is aggregated at low densities and regularly distributed at higher densities. Furthermore, Myers (1978) suggests a variance/mean ratio > 1 can indicate an aggregated distribution. Spatial aggregations of nymphs and adults are consistent with findings from previous studies conducted both in the insect's native and invasive range. Adults and nymphs of *M. cribraria* have been shown to have an aggregated distribution in India on pigeonpea, *Cajanus cajan* (L.) Millspaugh, based on Taylor's power law and Iwao's patchiness regression (Borah et al. 2002), and in Japan on soybean, based on the

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goodness of fit to the Poisson distribution (Kono 1990). Seiter et al. (2013a) also showed a clumped pattern of *M. cribraria* in South Carolina soybean fields, with a greater abundance along field edges.

The aggregated distribution of *M. cribraria* nymphs can be explained by the behavior of females ovipositing in clusters of two or three parallel rows with ~16 eggs per mass (Zhang et al. 2012). This implies that nymphs would initially be more aggregated before potential dispersal. This is also supported by visual observations of nymphs of *M. cribraria* aggregating at growing points and nodes of various field crops (Thippeswamy and Rajagopal 2005). Adults of *M. cribraria* have been observed in mating aggregations on host plants (Hibino and Ito 1983) but have the ability to disperse with strong flying ability (Ruberson et al. 2013). This could explain the more random distribution associated with adults in this study.

The development of sampling plans is affected by spatial distributions of insects as well as sampling method (Espino et al. 2008, Knutson et al. 2008, Reay-Jones et al. 2009). Greater numbers of samples are required to obtain accurate population estimates at higher levels of aggregation (Karandinos 1976). For both sampling methods and life stages, determining population estimates at low densities required intensive sampling even at lower levels of reliability (Fig 1). At low mean population densities and for all levels of precision for both sampling methods, more sample units were required for adults compared with nymphs, with the reverse at higher densities. For example, at 30% reliability, 573 and 66 sample units were required for adults and nymphs, respectively, when sampling at a density of one insect per 20 sweeps, but 16 and 20 sample units were required for adults

and nymphs, respectively, when sampling at a density of 30 insects per 20 sweeps. This difference is explained by Wilson (1985), where a low b parameter value does not necessarily equate to a uniform spatial distribution but could determine different levels of aggregation at different densities.

The association between sweep-net and beat-cloth counts varied by life stage (Table 2.1), with a better fit for regression models for adults compared with nymphs based on r^2 values. Sweep-net samples in soybean have shown a bias toward adults and late instar nymphs (Seiter et al. 2013a) which would reflect this better fit. We can predict that this bias may also occur with the beat-cloth method. First instars average 1.1 ± 0.1 mm in length (Zhang et al. 2012). Because the beat-cloth method requires enumeration on the floor under the soybean canopy, small newly hatched nymphs are difficult to visually count.

Sequential sampling plans have been developed for pest sampling in many crop systems (Hoffmann et al. 1991, Espino et al. 2008, Reay-Jones et al. 2009). Decision making using IPM requires classification of a population above or below a threshold rather than absolute population density estimation. Hence, sequential sampling plans for estimating densities for IPM generally require a lower number of sample units to reach the desired outcome. Sample size needed to reach a decision is greater when densities are close to the economic threshold. Sequential sampling plans are most efficient when infestation levels are well below or above economic thresholds. Plans were designed only for the immature stage of the insect because presence of nymphs in soybean fields has been associated with yield loss (Seiter et al. 2013b), and nymphs are currently the targeted life

stage for intervention (Greene et al. 2012). No thresholds relating to adult *M. cribraria* have been recommended.

A type I error would result in an unnecessary insecticide application to the crop, while a type II error would result in no intervention occurring when there should be, which could result in economic yield loss. It is important to balance α and β by determining sampling and insecticide application costs (α) and the damage cost due to yield loss (β) (Wilson 1994). Two error rates (0.1 and 0.2) in this study give a level of flexibility to each sampling plan. Different circumstances may require a more liberal or conservative approach to management of *M. cribraria*. For example, a high value crop or a sudden increase in insecticide application cost may require a lower risk strategy, with error rates $\alpha = \beta = 0.1$. Consequently, this increases the number of sample units required and, hence, the time costs associated with sampling. Other consequential costs associated with insecticide treatment, such as insecticide resistance development, environmental costs, and re-infestation of fields are also taken into account by α. In the short term, effective chemical applications are the most widely used, immediate option for control of exotic species, such as *M. cribraria*. Natural enemies often do not exist, resistant varieties are not available, and biological information concerning the pest at the time of invasion is often insufficient due to literature that is frequently printed in the language of the invader's native country (Bansal et al. 2013). Applications of pyrethroid insecticides are the only economically viable control available to reduce infestations and crop damage for *M. cribraria* (Ruberson et al. 2013). Issues with insecticide resistance have yet to be documented with *M. cribraria*, but resistance is reported extensively for many insects, with over 500 species resistant to

one or more insecticides (Whalon et al. 2008). Assigning equal values to α and β underlines the significant economic outcomes if resistance were to develop due to unnecessary applications of insecticide.

Cost-reliability analyses of various sampling methods of predatory arthropods on cotton, *Gossypium hirsutum* L., have been used to successfully enhance IPM programs (Parker et al. 2005, Knutson et al. 2008). Our cost-reliability analysis incorporated the time required to count the number of insects, which, as expected, increased as population density increased. When comparing the beat-cloth method with the sweep-net method, the beat-cloth method was the most cost-reliable method for all life stages and all densities for pest management. For population estimation, the sweep-net method was most effective at low densities of adults and nymphs, but, as densities increased, the beat-cloth method became more cost effective. Cost-reliability depends on sampling costs, insect density, and corresponding variance but does not rely on the economic threshold or error rates. Espino et al. (2008) enhanced their cost-reliability model comparing sweep-net sampling to visual sampling of *Oebalus pugnax* (*F*.) in rice, *Oryza sativa* L., by the addition of a variable for 'adoption of sampling strategy'. This is particularly important if scouts and consultants have an aversion to a particular method of sampling, even if it is the most cost-reliable method. In South Carolina, beat cloths are recommended for sampling many other soybean pests, with specific thresholds available for scouts, growers, and consultants for both hemipterans and lepidopterans (Greene 2014); hence, adoption of beat cloths to sample *M. cribraria* would not prove a new challenge. Beat cloths, on the other hand, are not suitable

for sampling in narrow-row soybeans (Pitre et al. 1987), so adoption of sweep-net sampling would be more practical in this circumstance.

Developing a sequential sampling plan with validation is crucial if it is to be used in the field. Currently, the sample size recommendation for *M. cribraria* in soybean is at least 10 sweeps at 10 locations in a field (Greene et al. 2012). A comparison of the fixed sample size plan with the sequential sampling plans developed in this study shows that significant savings in time are possible when utilizing sequential plans. Field-date combinations sampled ($n = 20$) with a fixed sample size of $n = 10$ had low densities of *M. cribraria* nymphs, and up to 60% reduction was achieved by using sequential sampling with the sweep-net and beat-cloth methods. These results echo other comparisons between fixed and sequential sampling plans with stink bugs in cotton in South Carolina and Georgia (Reay-Jones et al. 2009) and *O. pugnax* in rice in Texas (Espino et al. 2008).

Sampling plans for population estimation and pest management were developed for a new insect pest of soybean in the United States which integrate spatial distributions, economic thresholds, and designated levels of reliability. Our sequential sampling plans can improve efficiency with regards to the number of sample units required to reach management decisions in South Carolina. The beat cloth was the most cost-reliable sampling method to sample both adults and nymphs of *M. cribraria* across most population densities. These results may be used by researchers, county Extension agents, consultants, and farm managers to both facilitate sampling and improve reliability of *M. cribraria* estimates for research purposes and for IPM decision making.

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Table 2.1. Equation relating counts of *Megacopta. cribraria* nymphs using sweep-net sampling (independent variable) and beat-cloth sampling (dependent variable) to determine economic thresholds for development of sequential sampling plans for beat-cloth sampling. A threshold of one nymph per sweep was used to determine threshold.

Stage	Intercept ± SEM	Slope ± SEM	F	P	r^2	n	BC^a economic threshold ± SEM
Adult	1.881 ± 0.778	0.714 ± 0.088	62.62	<0.0001	0.483	68	-
Nymph	1.672 ± 0.944	1.155 ± 0.119	22.89	<0.0001	0.263	66	24.8 ± 4.606

[a] Beat-cloth

Table 2.2. Coefficients for Taylor's power law for *Megacopta cribraria* (adults and nymphs) sampled in eight fields in South Carolina during 2012 and 2013.

Sampling Method	Stage	*a*	*b*	r^2	t value for $b = 1$	Var: mean ratio
Sweep Net	Adult	18.998	0.901	0.251[a]	0.39NS	27.00
	Nymph	2.126	1.615	0.993[a]	20.20[b]	39.87
Beat Cloth	Adult	28.804	0.871	0.557[a]	1.03NS	42.76
	Nymph	2.973	1.620	0.970[a]	7.72[b]	48.33

NS, not significant
[a] $P < 0.0001$
[b] t-test indicates *b* is different from a value of 1 at $\alpha = 0.05$

Table 2.3. Comparison of mean sample size (±SEM) required to reach a management decision for the sweep-net and beat-cloth methods for both error rates ($\alpha = \beta = 0.1 = 0.2$) using the sequential sampling plan versus the fixed sampling plan in South Carolina soybean fields.

Method	Error rate ($\alpha = \beta$)	Mean sample size (±SEM)	% reduction	Decisions					
				Fixed			Sequential		
				No intervention	Continue sampling	Intervention required	No intervention	Continue sampling	Intervention required
Sweep net	0.1	4.1 ± 0.1	59	18	2	0	20	0	0
	0.2	4.0 ± 0.0	60	20	0	0	20	0	0
Beat Cloth	0.1	4.0 ± 0.0	60	20	0	0	20	0	0
	0.2	4.0 ± 0.0	60	20	0	0	20	0	0
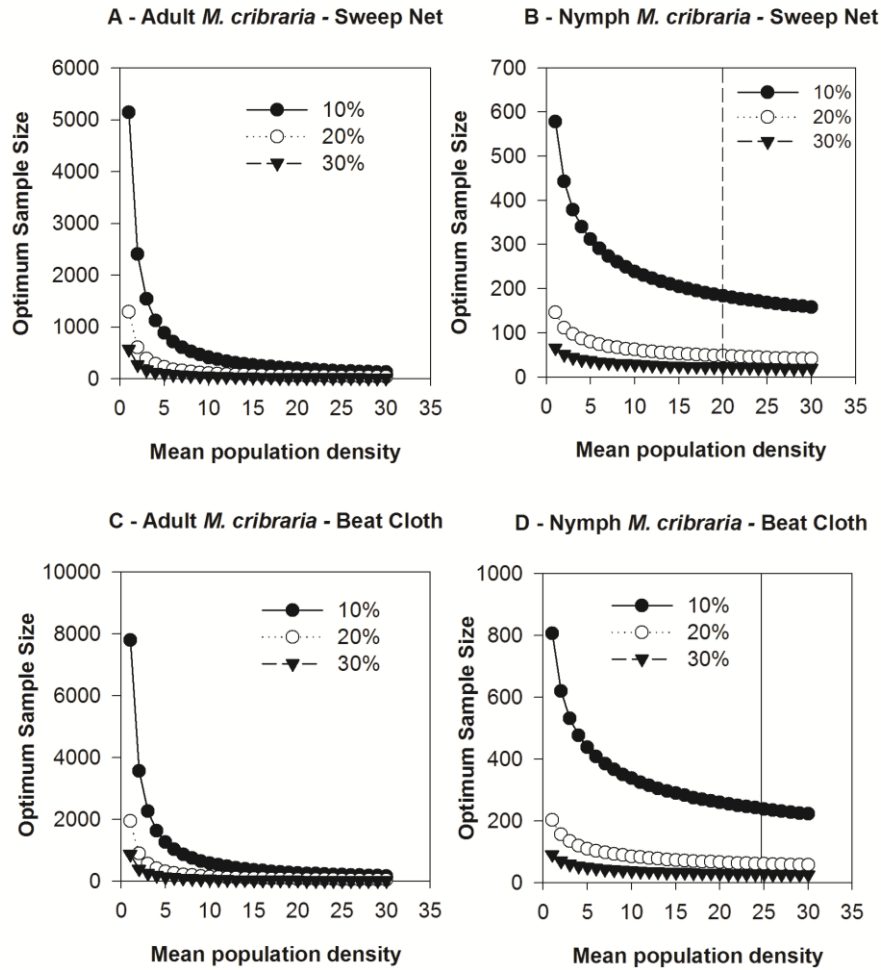
Fig. 2.1. Optimum sample size required to obtain population estimates within 10, 20, and 30% of the mean for (A) adult *Megacopta cribraria* with the sweep-net method, (B) nymph *M. cribraria* with the sweep-net method, (C) adult *M. cribraria* with the beat-cloth method and (D) nymph *M. cribraria* with the beat cloth method. Mean population density is expressed as number of *M. cribraria* per 20 sweeps or as number of *M. cribraria* per 1.83 m of row. Dashed vertical line represents the current recommended threshold of one nymph of *M. cribraria* per sweep (20 nymphs per 20 sweeps). Solid vertical line represents predicted economic threshold using the beat cloth (24.7 nymphs per 1.83 m of row).
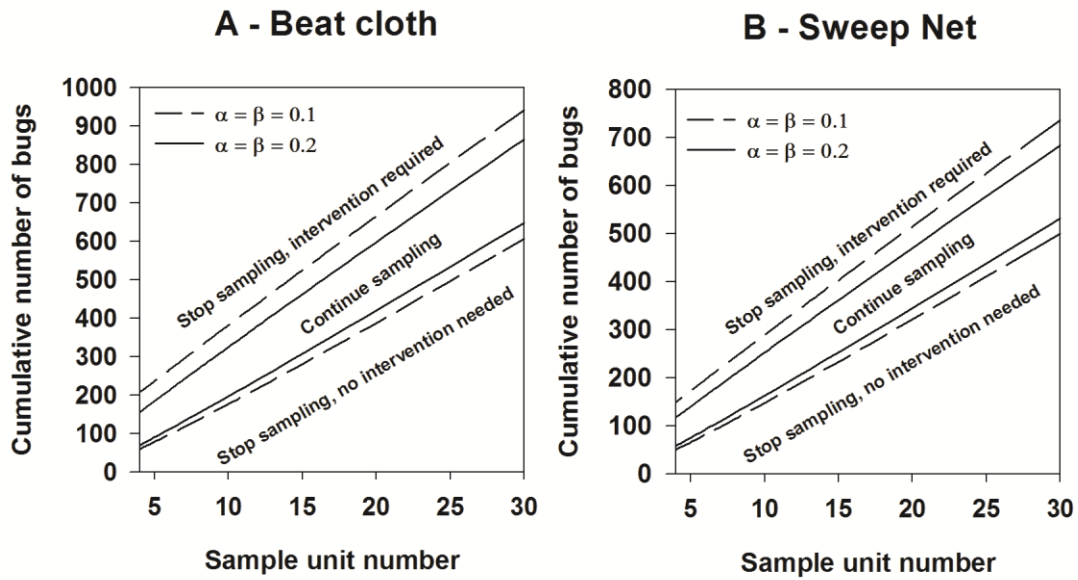
Fig. 2.2. Sequential sampling plan for nymphs of *M. cribraria* using (A) the beat-cloth method for an economic threshold of 24.7 nymphs per 1.83 m of row and (B) the sweep-net method for an economic threshold of one nymph per sweep (20 nymphs per 20 sweeps). Two sets of error rates were used $\alpha = \beta = 0.1$ and $\alpha = \beta = 0.2$.

A - Population Estimation
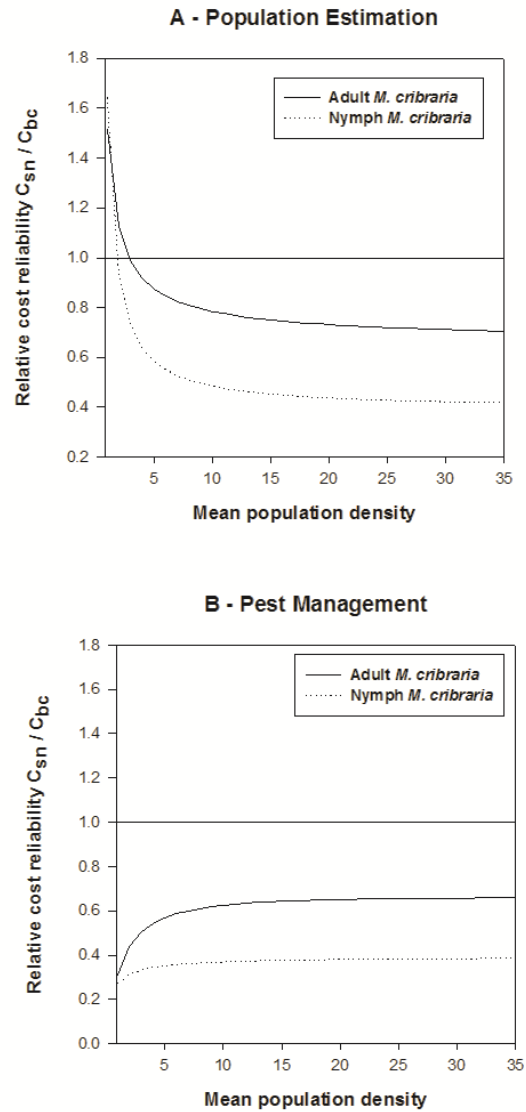
B - Pest Management

Fig. 2.3. Relative cost-reliability of the beat-cloth method for (A) population estimation and (B) pest management sampling with respect to sweep-net sampling in soybean. Mean population density is expressed as the number of *M. cribraria* per 20 sweeps. The straight line represents relative cost-reliability value of one, where the sweep net and the beat cloth have the same cost reliability. The beat-cloth method is more cost-reliable below the line (at y = 1); the sweep net is more cost reliable above the line.

CHAPTER III

ASSESSMENT OF A CROSS-VANE TRAPS AS A TOOL FOR SAMPLING THE

INVASIVE *Megacopta cribraria* (HEMIPTERA: PLATASPIDAE) IN SOYBEAN

WITH ASSOCIATED EVALUATIONS OF REPRODUCTIVE STATUS

First reported forming overwintering aggregations on and around homes in northeastern Georgia, United States in 2009, the invasive kudzu bug, *Megacopta cribraria* (F.) (Hemiptera: Plataspidae), has now been reported in thirteen other states and the District of Columbia (Suiter et al. 2010, Gardner et al. 2013). Originating from the most southwesterly of Japan's four main islands (Hosokawa et al. 2014), *M. cribraria* is only the second species from the family Plataspidae to occur west of the prime meridian (Beardsley and Fluker 1967, Eger et al. 2010). In addition to feeding on the invasive weed kudzu, *Puereria montana* (Loureiro) Merrill variety *lobata* (Willdenow), the insect also feeds on soybean, *Glycine max* (L.) Merrill, and is now an established pest throughout the southeastern United States (Seiter et al. 2013b, 2015).

Adults and nymphs use piercing-sucking mouthparts to feed on soybean stems, petioles, and leaves, but unlike closely related pentatomids, do not feed on seed or pods (Seiter et al. 2013b). Associated yield losses have been attributed to feeding-induced stress during soybean seed fill and reduced photosynthetic capacity due to feeding-induced necrotic lesions and sooty molds growing on *M. cribraria* honeydew secretions (Thippeswamy and Rajagopal 2005, Seiter et al. 2013b). From 2011 to 2014, over $6.7 million of yield loss plus insecticide treatment costs were attributed to *M. cribraria* in the southern United States (Musser et al. 2012, 2013, 2014, 2015). The economic damage to

soybean, lack of knowledge regarding this invasive species in its new range, and threat of a Central American enforced export trade embargo on shipments from the United States (Ruberson et al. 2013) motivated researchers to elucidate the biology and ecology (Zhang et al. 2012, Del Pozo-Valdivia and Reisig 2013), host range and preferences (Medal et al. 2013, Blount et al. 2015), spatial distributions (Seiter et al. 2013c), and management strategies for *M. cribraria* (Seiter et al. 2015).

In the southeastern United States, the kudzu bug has a bivoltine life cycle (Zhang et al. 2012). Adults emerge from overwintering sites in the early spring and move to kudzu, where females preferentially oviposit on growing vine tips (Zhang et al. 2012). First generation adults generally develop on kudzu and then move to soybean (Zhang et al. 2012, Seiter et al. 2013c) where females oviposit to produce the season's second and final generation. Overwintered adults may also colonize soybean directly, bypassing kudzu, if available early in the season (Del Pozo-Valdivia and Reisig 2013). The search for suitable overwintering sites occurs when temperatures decline and day lengths shorten (Zhang et al. 2012, Golec and Hu 2015). Reported overwintering sites include beneath loose pine, *Pinus* spp. (Lahiri et al. 2015), pecan, *Carya illinoinensis* (Wangenh.) K. Koch (Golec and Hu 2015), and sweet gum, *Liquidambar styraciflua* L. bark, beneath leaf litter (Lahiri et al. 2015), and inside residences (Ruberson et al. 2013).

Dispersal cues from kudzu or overwintering sites to soybean are unknown, although an overcrowding factor has been hypothesized (Zhang et al. 2012). Population dynamics of *M. cribraria* have been documented in the context of adult and nymph abundance in soybean fields in South Carolina (Seiter et al. 2013c) and Georgia (Blount et al. 2015,

Blount et al. 2016), but adult population structure represented by reproductive status has not been documented. Seasonal reproductive condition of hemipterans can provide valuable information about adult life history characteristics, which can influence pest management strategies (Kiritani 1963, Toscano and Stern 1980, Herbert and Toews 2011, Herbert and Toews 2012).

As with other soybean hemipteran pests (Rudd and Jensen 1977, Kogan and Pitre 1980, Todd and Herzog 1980), sweep-net sampling is the primary tool to sample both adults and nymphs of *M. cribraria* (Seiter et al. 2013c, Stubbins et al. 2014, Blount et al. 2015, Seiter et al. 2015). Sweep-net sampling involves swinging a 38-cm-diameter net at 180º across one or two soybean rows (Kogan and Pitre 1980) and is also the recommended monitoring and scouting technique for *M. cribraria* in soybean to classify whether the insect population is above or below the published threshold (Seiter et al. 2013a). Based on a design by Ulyshen and Hanula (2007), white cross-vane traps have been suggested as an alternative method for sampling and monitoring adults of *M. cribraria* (Horn and Hanula 2011). Adults are attracted to a white cross-vane, and a soapy water-filled bucket below the cross-vane captures flying insects as they encounter the vanes and fall. Zhang et al. (2012) used cross-vane traps to monitor adult activity in kudzu patches, but there are no studies evaluating white cross-vane traps in soybean fields as a sampling tool for decisions. The objectives of this study were (1) to determine the effect of distance from the field edge on densities of *M. cribraria* in soybean captured by cross-vane trap and sweep-net sampling, (2) to assess the association between cross-vane trap and sweep-net sampling in

soybean fields, and (3) to determine female population structure in soybean sampled through cross-vane traps and sweep-net sampling.

Materials and Methods

Cross-Vane Trap Design. Traps were constructed based on descriptions by Horn and Hanula (2011) but adapted to be raised above the soybean canopy. In brief, metal conduit (305 cm long, 1.9 cm thickness) was cut into two sections. The post (244 cm) was driven directly into the ground (about 61 cm). The support arm (61 cm) was affixed to the top of the post with a galvanized steel U-bolt, and tightened. The cross-vane was made by cutting grooves halfway down the middle of two white plastic corrugated boards (20-cm x 30-cm x 0.4-cm) and sliding them together to create a barrier with two intersecting planes. Holes punched at the top of each vane and in the support arm allowed assembly via a plastic coated vinyl clothesline wire and an S-hook. Holes punched at the bottom of each vane allowed for attachment to a 5.57 L capacity black bucket. Buckets were filled with soapy water (Dawn® soap, Proctor and Gamble, Cincinnati, OH, USA). Four small holes were drilled into the side of each bucket (13-cm from the bottom) to prevent overflow during rainfall.

Soybean Fields. Cross-vane trap and sweep-net sampling were conducted in five soybean fields in 2013 and 2014 at the Clemson University Edisto Research and Education Center (EREC) near Blackville, SC. Field size ranged from 3.6 to 7.3 ha with an average of 5.2 ± 0.3 (SEM) ha across all years. Soybean fields were planted on 97 cm rows.

Insecticides were not used for *M. cribraria* management, except on 17 August 2013 when *gamma*-cyhalothrin (Declare®; 0.017kg [AI] / ha) was applied to all five fields.

Soybean Sampling. In each field, four distances from the field edge (0, 10, 20 and 40 m) were marked with stakes within the row, on three transects (two at one field end, one at the opposite field end; 12 sampling locations per field). Each field was considered a replicate and each transect a sub-sample. A cross-vane trap was placed at each stake. Up to three times per week, insects were collected from the traps and returned to the laboratory. Individual counts of male and female adults were noted for a combined weekly total. Traps were refilled with soapy water as required. Each week, sweep-net sampling was conducted with twenty sweeps across two rows (ten sweeps on two rows adjacent to the stake and ten sweeps on the next two rows), moving back towards the stake for each sampling location (240 sweeps per field per week). Rows were sampled alternatively on each side of the stake each week to minimize crop damage. Sweep-net samples were collected in plastic bags (30 x 50 cm) and frozen (-20°C) in the laboratory before males, females, and nymphs were enumerated. In 2013, sampling was conducted from late vegetative growth (26 July; V8 [Fehr et al. 1971]) in three fields and full bloom (8 August; R2) in two fields. In 2014, sampling was initiated at V8 (30 June; 2 fields, 15 July; 3 fields). In 2014, pan-sampling was conducted as an alternative to sweep-net sampling in all fields until V8 (from V1 in two fields, V2 in one field, and V5 in two fields), as the plants were considered too small to be sampled with a sweep-net. Soybean plants were shaken over a white pan (40-cm by 32-cm; $n = 10$), as close as possible to each stake. Insects were counted in the field and

adults were not sexed for pan samples. In both years, sampling continued until R6 (full seed).

Dissections. In 2013, up to ten females, collected from sweep-net samples and cross-vane traps, were randomly selected for each soybean field, per week, and partially dissected by using a razor blade and fine forceps to remove the entire scutellum and pleural membrane to expose the internal organs (up to 50 females per week, per sampling method). All dissections were performed under 80% ethanol. In 2014, ten females collected from sweep-net sampling and cross-vane trap sampling were randomly selected from each soybean field at each of the four distances (0, 10, 20, 40-m) and partially dissected (200 females per week, per sampling method). Female reproductive organs were evaluated and given a rating of non-reproductive (Fig. 3.1A), intermediate (Fig. 3.1B), or reproductive (Fig. 3.1C), based on developmental stage; similar to previous work with the brown stink bug, *Euschistus servus* (Say) (Herbert and Toews 2011). Non-reproductive females had no visible eggs present in their ovaries and, hence, were either newly emerged or had already laid eggs. Intermediate females had ovaries that contained immature eggs with a soft chorion that were malleable when pressed with forceps. Reproductive females had mature eggs with a hardened chorion that filled the entire abdominal cavity. Reproductive organs were photographed with a zipScope 2M USB Digital Microscope (Aven Inc., MI).

Data Analysis. For each sampling method, in both years, an overall expected 1:1 ratio of males to females collected was tested using a χ^2 test. Male, female, total adult, and nymph counts from cross-vane traps and sweep-net sampling (pan sampling counts were disregarded due to low numbers) were analyzed separately for each year using a mixed

linear model approach (PROC MIXED, SAS Institute 2010), with distance, week, and the interaction as fixed effects. Random effects in the model included field, transect, distance and week interactions, all nested within field. Week was included in a repeated measures statement with a first order autoregressive covariance structure. Insect count data were log transformed ($\log_{10}[x + 1]$) before analysis (Zar 1999). Significant two-way interactions were further analyzed using the SLICE statement of PROC MIXED. Least-squares means were tested using pairwise t-tests ($\alpha = 0.05$) with the Tukey-Kramer adjustment (Tukey 1949). Furthermore, because distance is a continuous variable, a trend analysis was conducted to interpret significant distance effects using orthogonal contrasts. The PROC IML function was used to obtain coefficients for the contrasts for unequally spaced data (0, 10, 20, and 40-m)

The strength of the linear association (correlation) between cross-vane trap count and sweep net count was measured with the Pearson correlation coefficient (r) (JMP 2013). Data were analyzed across both years, across all distances from the field edge and week and then separately for each year, distance from the field edge, and week.

As the number of female samples available to dissect per location differed over the course of the season, reproductive ratings (non-reproductive, intermediate, and reproductive) were calculated as proportions observed per week. The Cochran-Mantel-Haenszel test was used to compare the proportion of each reproductive rating collected by sweep-net and cross-vane trap sampling across the season (JMP 2013).

Results

Cross-Vane Trap Sampling. In 2013, 550 adults (282 female, 268 male) were collected from cross-vane traps over nine weeks [1.1 ± 0.1 (SEM) per trap per week]. The sex ratio did not differ from an expected ratio of 1:1 ($\chi^2 = 0.356$; df = 1; $P = 0.5505$). In 2014, 7,052 adults (3,409 female, 3,643 male) were collected in cross-vane traps, over 15 weeks (7.9 ± 0.4 per trap per week). The sex ratio was significantly biased towards males ($\chi^2 = 7.765$; df = 1; $P = 0.0053$). The maximum number of adults caught in one cross-vane trap was 116 on 20 September 2014 (soybean stage; R6, located on the field edge), corresponding to 411 adults per 20 sweeps (20.6 per sweep).

Distance from the edge of the field affected the number of females and total adults in 2013 and males and total adults in 2014 (Table 3.1, Fig. 3.2A and Fig. 3.2B). In 2013, trend analyses of the distance effect (Table 3.1) indicated linear trends for females ($F = 14.35$, df = 1, 42, $P = 0.0005$) and total adults ($F = 11.48$, df = 1, 42, $P = 0.0015$). In 2014, trend analyses of the significant distance effects showed linear trends for total adults ($F = 15.72$, df = 1, 42, $P = 0.0003$) and males ($F = 19.18$, df = 1, 42, $P < 0.0001$). The linear trends observed were characterized by the significantly higher counts at 0 m compared to 40 m (Fig. 3.2A and Fig. 3.2B). Week only affected the number of males captured in 2013 (Table 3.1, Fig. 3A). Adult numbers sampled by the cross-vane trap in 2013 remained very low over the entire sampling period (Fig. 3.3A). Week influenced the numbers of males, females, and total adults in 2014, showing a steady increase to a peak from initial sampling date in June until 6 September, followed by a decline in numbers until the last sampling date (Table 3.1, Fig. 3.3B). Analysis of slice effects of all significant week-distance

interactions (Table 3.1) highlighted variability in the decrease in trap capture from 0 to 40 m in both years (Fig. 3.4A and Fig. 3.4B).

Sweep-Net Sampling. In 2013, 1,187 adults (533 female, 654 males) were collected by sweep-net sampling over 12 weeks (2.6 ± 0.54 adults per 20 sweeps per week). In 2014, 12,078 adults (5,886 female, 6,192 male) were collected by sweep net sampling, over 12 weeks (30.3 ± 15.8 per 20 sweeps per week). In both years, the overall sex ratio was biased towards males for sweep-net captures (2013; $\chi^2 = 12.334$; df = 1; $P = 0.0004$, 2014; $\chi^2 = 7.753$; df = 1; $P = 0.0054$). The maximum number of adults per 20 sweeps was 473 (26 September 2014; R6), with a corresponding 61 adults captured per trap at 0 m from the edge of the field. A total of 427 (mean 0.79 ± 0.21 per 20 sweeps per week) and 8,440 (mean 15.8 ± 5.5 per 20 sweeps per week) nymphs of all instars were collected in 2013 and 2014, respectively.

Analyses of main effects generated results with similar patterns to cross-vane trap sampling. Distance affected the number of males (2013 and 2014) and total adults (2014) (Table 3.1, Fig. 3.2C and Fig. 3.2D). Trend analyses of the distance effect indicated linear trends for males in 2013 ($F = 4.79$, df = 1, 42, $P = 0.0342$) and in 2014 ($F = 19.87$, df = 1, 42, $P < 0.0001$) and for adults in 2014 ($F = 11.51$, df = 1, 42, $P = 0.0015$). Linear trends were characterized by a decrease in counts along transects from the field edge (0 m) to 40 m (Fig. 3.2C and Fig. 3.2D). In 2013, counts of males, females, adults, and nymphs were all influenced by week. Adult numbers reached a peak on 2 August and decreased over the following two-week period (Fig. 3.3A). Following insecticide application on 17 August, adult numbers began to gradually increase until the last sampling date. Nymphs were first

collected in the field on 9 August, three weeks after sampling began. Numbers of nymphs gradually increased through the season. In 2014, week influenced all life stages sampled, as in 2013 (Fig. 3.3B). Sampling started earlier in 2014 and highlighted initial soybean colonization by adult *M. cribraria*. Adult numbers remained low until 19 August when numbers rapidly increased to reach 196 adults per 20 sweeps. Nymphs were observed in the field four weeks after the first adult was collected in sweep-net samples and followed a similar trajectory to that of adult numbers in 2014 (Fig. 3.3B). Analysis of the slice effect at all significant distance-week interactions in 2014 showed variability in the decrease in adults from the field edge (0 m) towards the interior of the field (40 m) (Fig. 3.4C).

Relationship Between Cross-Vane Trap and Sweep-Net Sampling. Overall linear correlation analysis across both years revealed a highly significant positive association between trap and sweep-net sampling ($r = 0.60$, n = 1126; $P < 0.0001$). Of the 88 date-distances combinations analyzed, 32 (36%) resulted in significant associations between trap and sweep-net sampling, which were at distances of 0 m (25%), 10 m (18.7%), 20 m (31.3%) and 40 m (25%).

Female Population Structure. In 2013, totals of 221 and 145 females were dissected from cross-vane traps and sweep-net samples, respectively. In 2014, 1,643 and 1,486 females were dissected from cross-vane trap sampling and sweep-net sampling, respectively. There were no significant differences among distances from the field edge for the proportion of each reproductive status for sweep-net (non-reproductive: $\chi^2 = 0.230$, df = 1, $P = 0.6313$; intermediate: $\chi^2 = 0.968$, df = 1, $P = 0.3252$; reproductive: $\chi^2 = 0.05$, df = 1, $P = 0.8118$) and trap sampling (non-reproductive; $\chi^2 = 2.515$, df = 1, $P = 0.1127$,

intermediate; $\chi^2 = 0.088$, df = 1, $P = 0.7662$; reproductive; $\chi^2 = 3.07$, df = 1, $P = 0.0795$). Therefore, proportions were combined over distances in soybean fields in 2014, reflecting the sampling procedure in 2013.

Females of all three reproductive stages were present in sweep-net or cross-vane samples during 2013 (Fig. 3.5A and Fig. 3.5B) and 2014 (Fig. 3.5C and Fig. 3.5D). In 2013, cross-vane traps sampled a higher proportion of intermediate ($\chi^2 = 9.49$, df = 1, $P = 0.0021$) and reproductive females ($\chi^2 = 32.54$, df = 1, $P < 0.0001$) (Fig. 3.5A), while captures in sweep-nets yielded a greater proportion of non-reproductive females ($\chi^2 = 75.73$, df = 1, $P < 0.0001$) (Fig. 3.5B). Sampling week significantly influenced the proportions of non-reproductive ($\chi^2 = 18.30$, df = 1, $P = 0.0107$), intermediate ($\chi^2 = 33.95$, df = 1, $P < 0.0001$), and reproductive females ($\chi^2 = 34.71$, df = 1, $P < 0.0001$) collected from cross-vane traps. Sampling week significantly influenced the proportions of non-reproductive females ($\chi^2 = 35.08$, df = 1, $P < 0.0001$) and reproductive females ($\chi^2 = 27.15$, df = 1, $P = 0.1950$) but did not affect the proportion of intermediate females ($\chi^2 = 9.89$, df = 1, $P < 0.0001$) sampled by the sweep-net, although there was no obvious, distinct pattern of reproductive change for both sampling methods.

In 2014, a higher proportion of reproductive females ($\chi^2 = 54.30$, df = 1, $P < 0.0001$) (Fig. 3.5C) were captured in the cross-vane traps, while a higher proportion of non-reproductive ($\chi^2 = 7.43$, df = 1, $P = 0.0064$) and intermediate females ($\chi^2 = 49.83$, df = 1, $P < 0.0001$) (Fig. 3.5D) were captured in the sweep-nets. Week influenced the proportion of non-reproductive ($\chi^2 = 933.65$, df = 1, $P < 0.0001$), intermediate ($\chi^2 = 53.97$, df = 1, $P < 0.0001$) and reproductive ($\chi^2 = 859.96$, df = 1, $P < 0.0001$) females sampled with cross-

vane traps. Similarly, week influenced the proportion of non-reproductive ($\chi^2 = 900.62$, df = 1, $P < 0.0001$), intermediate ($\chi^2 = 262.70$, df = 1, $P < 0.0001$), and reproductive ($\chi^2 = 604.33$, df = 1, $P < 0.0001$) females collected by the sweep net. A well-defined pattern of change of reproductive status was observed in 2014, for both cross-vane trap and sweep-net sampling, with a gradual reduction in the proportion of reproductive females and an increase in the proportion of non-reproductive females collected during the course of the sampling period. In August and September, a large proportion of the sweep-net population consisted of non-reproductive females, corresponding with the female population peak observed from sweep-net sampling (Fig. 3.3B).

Discussion

Adults of *M. cribraria* were highly attracted to white cross-vane traps, as observed by Zhang et al. (2012) and Horn and Hanula (2011). Adults were captured in soybeans during 24 sampling weeks over two growing seasons. Zhang et al. (2012) reported trap catches of up to 2,000 adults in kudzu during a single week, while traps operated by Horn and Hanula (2011) collected an average of 60 adults per trap per week over the course of seven weeks in kudzu. Our study reported a maximum of 116 adults in one trap during the week of 6 August 2014 and a two-year average of 5.8 ± 0.3 per trap per week. Cross-vane traps were assumed to record dispersal into soybean fields from other hosts. Terminology describing insect movements varies widely (Dingle 2014). Terms such as migration and dispersal have been used interchangeably, without definition, within the literature describing *M. cribraria* (Zhang et al. 2012, Ruberson et al. 2013, Seiter et al. 2013c, Lovejoy and Johnson 2014). We followed Schneider (1962) who defined 'dispersal' simply

as movement that results in an increase in the mean distance between individuals. Local dispersal is critical to pest population dynamics, and an understanding of the factors involved can contribute to enhanced monitoring and management tactics (Stinner et al. 1983, Kennedy and Storer 2000).

Distance from the field edge affected the total number of adults sampled from cross-vane traps (2013 and 2014) and sweep-net sampling (2014) (Fig. 3.2). Overall trends showed a linear pattern of decrease from the field edge (0 m) towards the interior of the field (40 m). This observation is not uncommon with other agricultural pests that fly between host plants (Tillman et al. 2009, Reay-Jones 2010, Reay-Jones et al. 2010). Indeed, spatial analyses of *M. cribraria* distributions in South Carolina reported that adult aggregation clusters were more likely to be located at soybean field edges compared with interior field locations (Seiter et al. 2013c). Our study supports this account but defines field edge at a finer level than Seiter et al. (2013c) where interior sampling began at approximately 35 m from the field edge. Distance from the field edge did not significantly influence the number of nymphs sampled, also supporting previous studies conducted in South Carolina (Seiter et al. 2013c).

Knowledge that adults of *M. cribraria* are present at greater numbers on the field edge has implications for research and management purposes. Sampling at the field edge will be less labor intensive and more productive if the objective is to collect high insect numbers for further research in the laboratory or to establish colonies. For pest management decision making, sweep-net samples executed both at the field edge and into the field interior will ensure that the sample is representative of whole-field populations.

Conversely, if nymphs are the focus of pest management decisions, sampling into the interior of the field may not be necessary, as distance into the field did not affect densities of immatures. Furthermore, adult aggregation along the edge of fields can be exploited for precision targeted insecticide applications such as those directed at the border of a field, if adults are to be targeted. This approach can reduce costs and pesticide usage and preserve natural enemies (Midgarden et al. 1997).

Although our study was not designed to test early season dispersal into soybean, we observed adults in cross-vane traps up to three weeks before the first adult was collected using pan or sweep-net sampling in 2014 (Fig. 3.3B). There was no relationship between numbers of insects captured with successive early trap catches and cumulative field populations estimated by sweep-net sampling at the end of the season (analysis not shown); hence, early season trap captures represented the presence of *M. cribraria* and the timing of population establishment, but they did not predict cumulative field populations, at least those indicated by the sweep-net sampling method. Cullen and Zalom (2005) showed that early season catches of the consperse stink bug, *E. conspersus* (Uhler), in pheromone-baited traps correlated with the overall cumulative population in tomato, *Solanum lycopersicum* L., sampled with sweep nets. The authors suggested that early season trap capture, as in our study, could be used as a biofix for a degree-day model. Studies of laboratory rearing have reported temperature dependent egg incubation and nymph development durations for *M. cribraria* (Del Pozo-Valdivia and Reisig 2013). Current guidelines suggest intervention when nymphs of *M. cribraria* reach one per sweep (Seiter et al. 2015); the use of cross-vane traps as a biofix, combined with egg and nymph

developmental rates and average temperatures during the year, could be used to delay labor intensive scouting until predicted nymph hatch.

Monitoring for insect pests is a critical component of integrated pest management programs, as it provides a basis for targeting populations when they exceed an economic threshold (also called an action threshold) as opposed to prophylactic or calendar based treatment thresholds. Monitoring and scouting using sweep nets can be time-consuming and labor-intensive (Stubbins et al. 2014). By comparison, a monitoring trap would provide consistent and standardized information that relate to insect densities in the field (Suckling 2000). A major limitation of using cross-vane traps for monitoring adults of *M. cribraria* in soybean is the lack of a consistent relationship between cross-vane trap and sweep-net catches (representing the within-field population). For example, in August 2013 and 2014, cross-vane trap capture was not associated with sweep-net capture convincingly, whereas, in September, sweep-net counts were associated with cross-vane trap counts, but ranged from 0.5–9.8 times as large as cross-vane trap catches, depending on sampling location and week. Reevaluations of current action thresholds (Seiter et al. 2015) are being considered, and, although not a consistent monitoring tool throughout the season, cross-vane traps may have value, in part, if revised action thresholds include early season evaluations of adults. Response to traps enhanced by pheromones have been used in hemipteran IPM programs as monitoring devices (Cullen and Zalom 2005, Borges et al. 2011). Isolation and exploitation of pheromones for *M. cribraria* may improve cross-vane trap interpretation in relation to actual field populations, as indirectly assessed by sweep-net sampling.

Soybean field infestation in June and July, field reinfestation after insecticide application, and continuous dispersal into fields have been previously documented (Zhang et al. 2012, Seiter et al. 2013c, Blount et al. 2015, Seiter et al. 2015), but this is the first study to report the female population structure of *M. cribraria* in soybean fields. Females of all reproductive stages were captured using sweep nets and cross-vane traps. Our sampling and dissection methods provided a means to illustrate the changes in seasonal dynamics with respect to female reproductive biology in the *M. cribraria* population in soybean fields. Non-reproductive females of *M. cribraria* disperse from overwintering sites to legume hosts where egg development begins followed by oviposition in the same host (Zhang et al. 2012, Golec and Hu 2015). Although there are two generations per year in the southeastern United States, the percentage of overwintered, first generation, or second generation adults dispersing to soybean is unknown. These data indicated that female populations dispersing into soybean fields may be a mixture of both newly emerged first generation and overwintered post-reproductive females who may have oviposited on a host such as kudzu. Overlapping generations, occupying the same location, is not uncommon in long-lived, multivoltine hemipterans (Kiritani 1963, Toscano and Stern 1980), and dispersal of reproductive females has been observed in other hemipterans, such as tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Stewart and Gaylor 1991) and *E. conspersus* (Cullen and Zalom 2006). Here, observations demonstrate that initiation of dispersal in *M. cribraria* is unlikely to be a constant life-cycle trait related to reproductive status (Fig. 3.5). A specific location characteristic, such as overcrowding, could be expected to be a dispersal initiator. Indeed, nymph peaks reached over a 1000 per

ten sweeps on 26 July 2013 in a kudzu patch approximately 12 km from EREC (FLS unpublished data).

Adults collected in sweep nets were either those that dispersed into soybean fields from other areas or those that developed from eggs deposited in sampled fields. Detection of adults in the cross-vane traps were predominantly individuals that dispersed into the soybean field, although there may be sampling of individuals that move within soybean (trivial dispersal) or that disperse out of soybean to alternate hosts. This may explain the difference in female population structure sampled by the sweep net and cross-vane trap. Continuous dispersal throughout the soybean growing season has implications for pest management, but, at present, dispersal ability and flying behavior of *M. cribraria* are poorly understood. Evaluations of flight activity using flight mills (Stewart and Gaylor 1994, Lee and Leskey 2015, Attisano et al. 2015), combined with field mark-recapture studies (Perfect et al. 1985, Alyokhin and Ferro 1999), will be essential to assess dispersal capacity of overwintered, first generation, and second generation populations relative to reproductive status and host plant. Information regarding potential dispersal distances could help identify soybean fields that are at risk of infestation and would aid predictions of spread to other soybean-growing states in the United States. Furthermore, understanding the vertical distributions of flying *M. cribraria* may be important for the optimal placement of cross-vane traps for monitoring purposes. Trap height above the canopy has been shown to be a factor associated with trap effectiveness for other insect species (Cottrell et al. 2000, Byers 2011). An overall, clearer understanding of where adults are dispersing from will also enhance our ability to exploit the biology and behavior for management purposes.

This study is the first to provide information about the use of cross-vane traps in soybean fields. Although effective at capturing adults of *M. cribraria*, cross-vane traps are limited to qualitative detection of populations rather than a precise sampling tool for population estimation. Additional studies are needed to determine their practical use in soybean for monitoring and sampling purposes. Those investigations should focus on flight behaviour of *M. cribraria*. Cross-vane traps may have value as an early monitoring tool in soybean-growing locations. This study is the first to rate the reproductive status of *M. cribraria* females sampled in soybean. Information regarding the population structure of dispersing female *M. cribraria* has improved the understanding of temporal dispersal patterns and provides a foundation for understanding dispersal cues to develop management strategies that may exploit dispersal behavior.

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Table 3.1. ANOVA *F*-statistics for the effects of week, distance, and the interaction on *Megacopta cribraria* counts in 2013 and 2014 from cross-vane trap sampling and sweep-net sampling, near Blackville, SC.

Sampling Method	Fixed effect	2013				2014			
		Males	Females	Total Adults	Total Nymphs	Males	Females	Total Adults	Total Nymphs
Cross-vane sampling	Distance[1]	2.24NS	5.18*	4.08*	-	6.97**	2.15NS	5.87*	-
	Week[2, 3]	2.67*	1.76NS	1.88NS	-	30.42***	19.42***	33.66***	-
	Interaction [4,5]	1.18NS	2.30**	1.76*	-	1.58*	0.86NS	1.22NS	-
Sweep-net sampling	Distance[1]	3.28*	1.47NS	2.75NS	0.18NS	6.63**	2.21NS	4.19*	2.13NS
	Week [2, 6]	6.28***	9.42***	8.82**	4.49**	21.06***	22.57***	22.89***	42.89***
	Interaction [4, 7]	1.24NS	0.55NS	1.08NS	1.11NS	1.71*	1.34NS	1.72**	1.36NS

NS, not significant. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.
[1] df = 3, 42
[2] df (2013) = 8, 100
[3] df (2014) = 14, 196
[4] df (2013) = 24, 300
[5] df (2014) = 42, 584
[6] df (2014) = 11, 133
[7] df (2014) = 33, 397

Fig. 3.1. Observed maturity of *Megacopta cribraria* reproductive organs. (A) non-reproductive female, (B) intermediate female, (C) reproductive female.
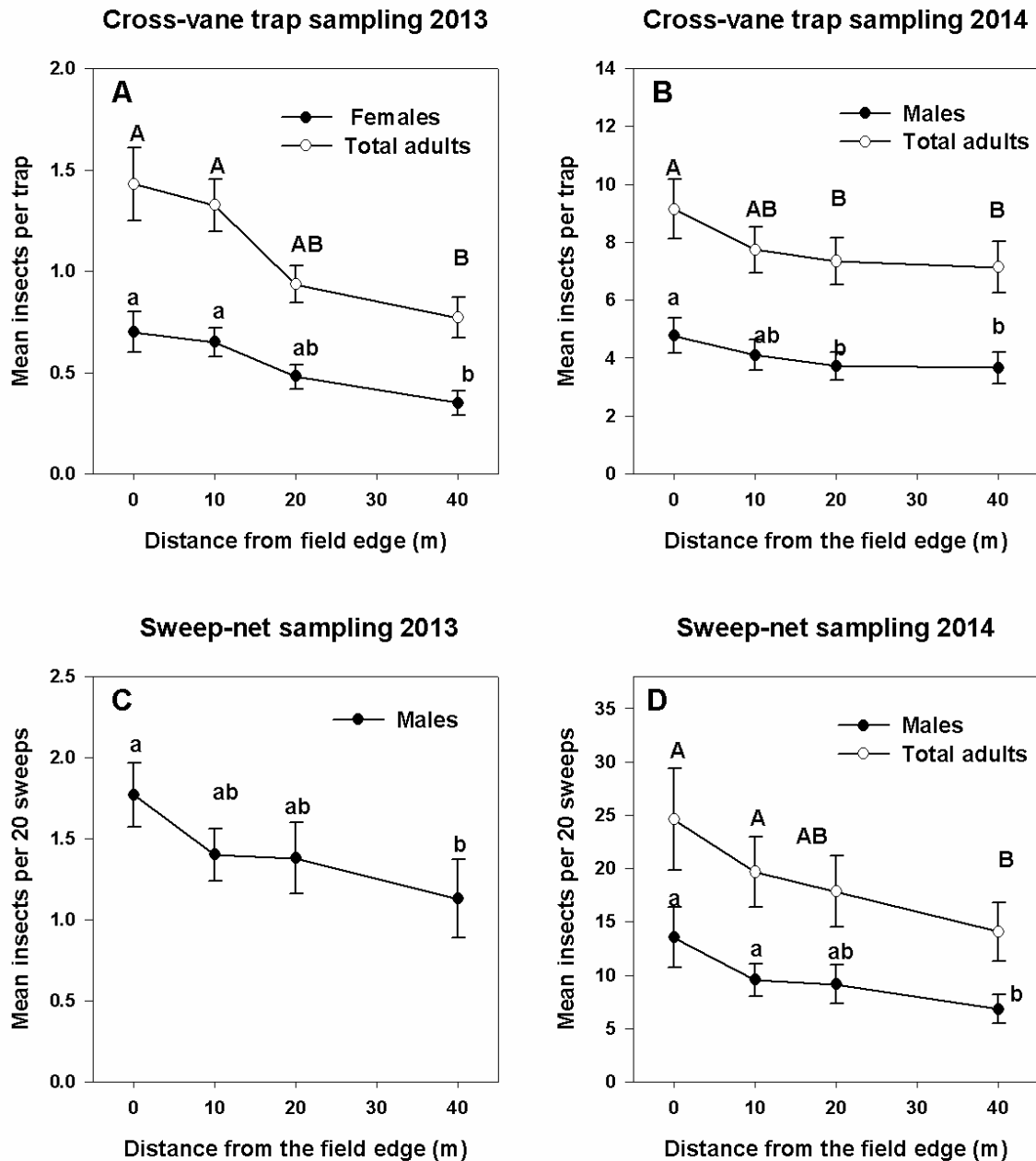
Fig 3.2. Mean (± SEM) *Megacopta cribraria* adult counts by distance from the field edge in (A) cross-vane trap sampling in 2013, (B) cross-vane trap sampling in 2014, (C) sweep-net sampling in 2013, and (D) sweep-net sampling in 2014. Different letters indicate significant differences based on Tukey-Kramer comparisons ($\alpha = 0.05$).

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Cross-vane trap and sweep-net sampling 2013   Cross-vane trap and sweep-net sampling 2014
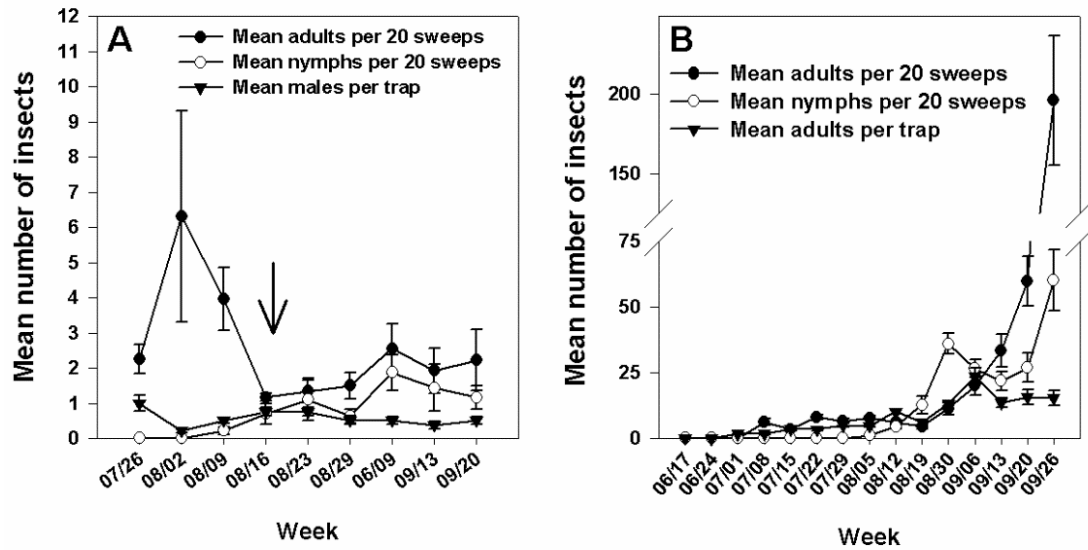
Fig. 3.3. Mean (± SEM) *Megacopta cribraria* counts by week across five soybean fields in Blackville, SC, using cross-vane traps and sweep-net sampling in (A) 2013 and (B) 2014. For simplicity, only select significant week effects are shown for each sampling method (Table 3.1). Arrow indicates insecticide application.
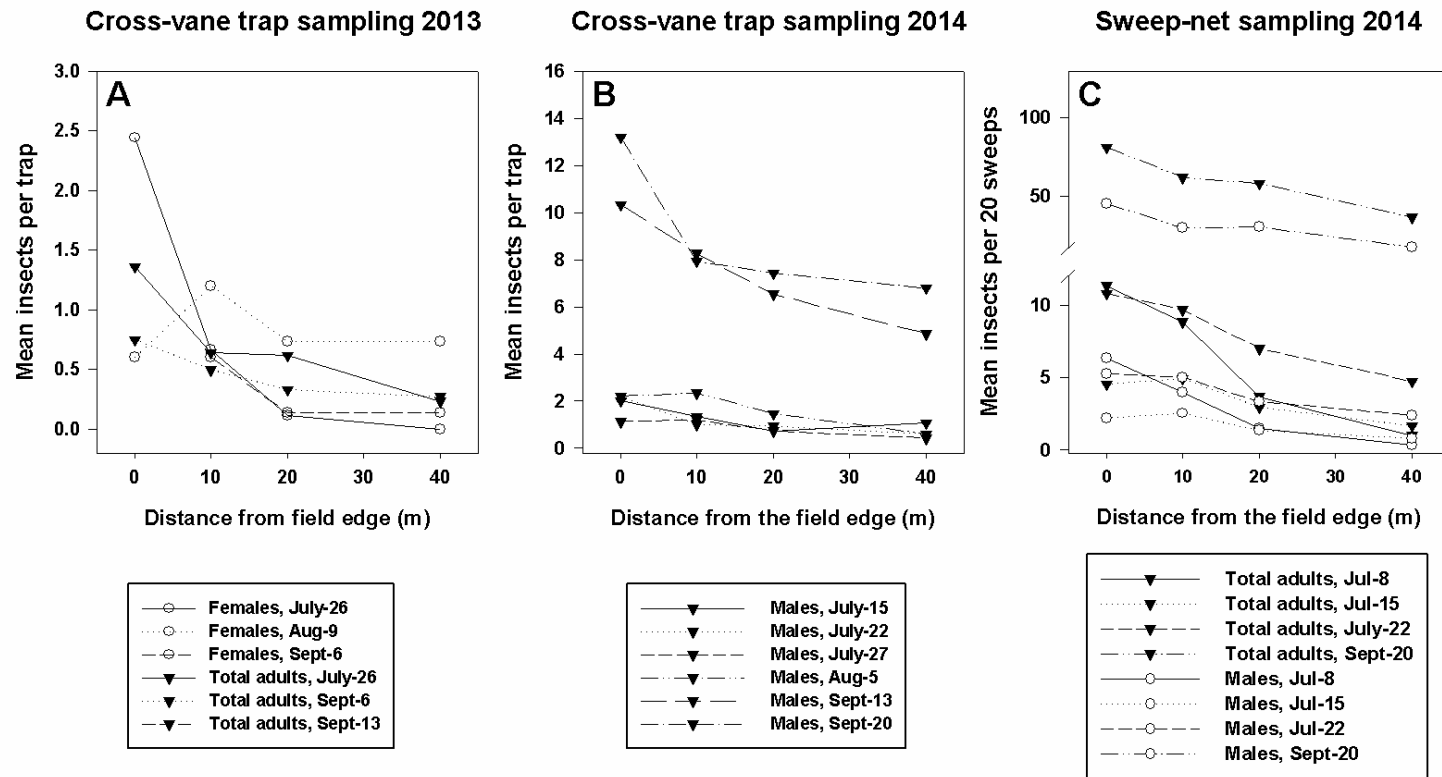
Fig. 3.4. Effect of distance from field edge on *Megacopta cribraria* adult counts from significant distance effects at each week (SLICE analysis of PROC MIXED) for (A) the cross-vane trap in 2013 on 26 July (*P* < 0.0001), 9 August (*P* = 0.0318), and 6 September (*P* = 0.0399) and for total adults on 26 July (*P* < 0.0001) and 9 September (*P* = 0.0330); (B) the cross vane trap in 2014 for males on 15 July (*P* = 0.0191), 22 July (*P* = 0.0004), 27 July (*P* = 0.0531), 5 August (*P* = 0.0060), 13 September (*P* = 0.0072), and 20 September (*P* = 0.0002); (C) the sweep net in 2014 on 8 July (*P* < 0.0001), 15 July (*P* = 0.026), and 20 September (*P* = 0.0312) for total adults and 8 July (*P* < 0.001), 15 July (*P* = 0.0223), 22 July (*P* = 0.0307), and 20 September (P = 0.0004) for males. SEM bars have been omitted for clarity.
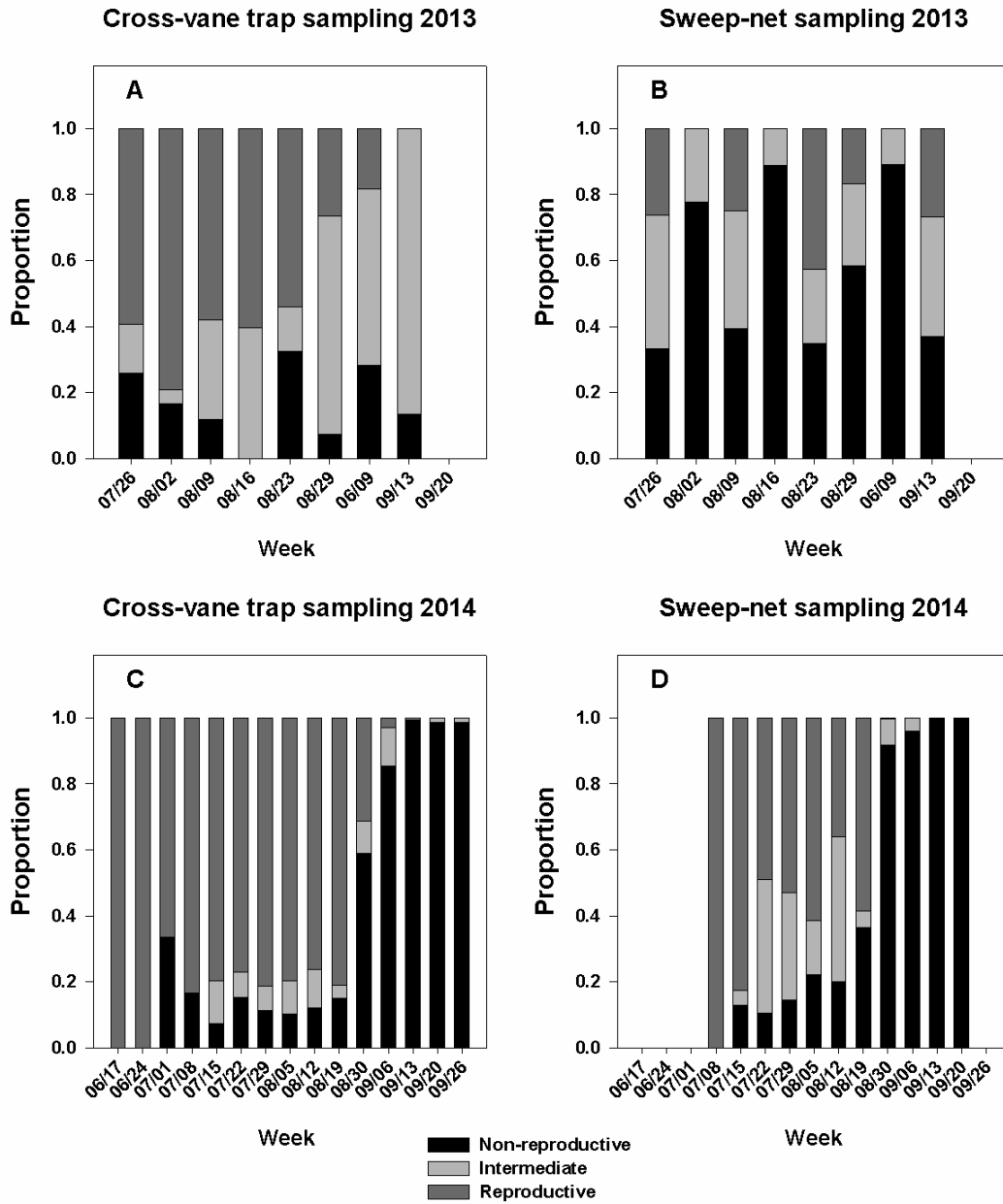
Fig. 3.5. Proportional populations of non-reproductive, intermediate, and fully reproductive *Megacopta cribraria* females observed by week from (A) cross-vane sampling in 2013, (B) sweep-net sampling in 2013, (C) cross-vane sampling in 2014, and (D) sweep-net sampling in 2014.

CHAPTER IV

SEASONAL ACTIVITY AND FEMALE POPULATION STRUCTURE OF THE

INVASIVE *Megacopta cribraria* (HEMIPTERA: PLATASPIDAE) IN KUDZU IN

SOUTH CAROLINA

Megacopta cribraria (F.) (Hemiptera: Plataspidae) was first discovered in northeastern Georgia in 2009 aggregating near and on the perennial weed kudzu, *Pueraria montana* Loureiro (Merrill) variety *lobata* (Willdenow) Maesen & S. Almeida (Suiter et al. 2010). This was the first time this insect species occurred in the Western Hemisphere (Eger et al. 2010). Although the pathway responsible for US introduction remains undetermined, mitochondrial haplotype analysis has indicated the most southwesterly of Japan's four main islands was the invasion origin (Hosokawa et al. 2014). Kudzu, also a native to Asia, was intentionally introduced into North America at the 1876 Centennial Exposition in Philadelphia, Pennsylvania. Initially, kudzu was distributed through mail-order catalogues where it was advertised as an ornamental vine, porch shade provider, and livestock forage crop. Seeds were also disseminated by the US Soil Erosion Service, for planting and establishing kudzu as a soil stabilizer (Forseth and Innis 2004). In 1997, kudzu was officially placed on the Federal Obnoxious Weed List due to its characteristic uncontrollable spread, facilitated by biased resource allocation to growth and a high photosynthetic rate (Forseth and Innis 2004). The leguminous weed now infests over three million hectares of the southern US (Forseth and Innis 2004). As a primary developmental host of *M. cribraria* (Zhang et al. 2012), this vast distribution of kudzu has contributed to

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the extensive US range of *M. cribraria* which extends to thirteen states and the District of Columbia (Gardner 2016).

In the southeastern US, *M. cribraria* has a bivoltine life cycle (Zhang et al. 2012). Adults emerge from overwintering sites in spring and can fly to kudzu where females preferentially oviposit on growing vine tips (Zhang et al. 2012). Adults of the overwintering generation can colonize soybean initially if available early in the season (Del Pozo-Valdivia and Reisig 2013). Nymphs undergo five molts before emerging as first-generation adults. The first generation may then colonize soybean (Seiter et al. 2013a), where females once again oviposit to produce the season's second generation (and hence incoming overwintering generation). If dispersal does not occur, the second generation can develop on kudzu (Zhang et al. 2012). The search for suitable overwintering sites begins in September (Zhang et al. 2012, Golec and Hu 2015). Reported overwintering sites include beneath loose bark of pine, *Pinus* spp. L., (Lahiri et al. 2015), pecan, *Carya illinoinensis* (Wangenh.) Koch (Golec and Hu 2015) and sweet gum, *Liquidambar styraciflua* L., beneath leaf litter (Lahiri et al. 2015) and inside residences (Ruberson et al. 2013). Dispersal cues from kudzu to soybean are unknown, although overcrowding has been hypothesized as a factor (Zhang et al. 2012). Other legumes have been classified as *M. cribraria* developmental hosts, such as pinto bean, *Phaseolus vulgaris* L., and pigeon pea, *Cajanus cajan* L. (Eger et al. 2010, Blount et al. 2015, Medal et al. 2013) and adults and nymphs can feed on other non-leguminous plants (Eger et al. 2010, Lovejoy and Johnson 2014).

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Adults and nymphs use piercing-sucking mouthparts to feed primarily on stems of host plants. Feeding on kudzu results in biomass reduction of up to 33% (Zhang et al. 2012) but *M. cribraria* can also cause yield loss in soybean (Seiter et al. 2013b). From 2011 to 2014, over $6.7 million in yield loss plus insecticide treatment costs was attributed to *M. cribraria* in soybean in the southern US (Musser et al. 2012, 2013, 2014, 2015). This figure is an underestimate, as Georgia and South Carolina were not included in the statistics. Soybeans were the second most abundant crop grown in the US in 2015, with a nationwide value of $34.5 billion (USDA-NASS 2016); hence the pest status of *M. cribraria* underlines the need to monitor and document abundance and population dynamics on primary developmental hosts and to determine patterns associated with host colonization and dispersal between hosts. Population dynamics and seasonal abundance of *M. cribraria* have previously been documented in the context of adult, nymph, and egg abundance in one patch of kudzu in Georgia in 2010 and 2011 (Zhang et al. 2012), but no studies on population structure represented by reproductive status in kudzu have been reported. Historically, it has been suggested that assessments of seasonal reproductive condition can predict patterns of population dynamics in hemipterans such as oviposition and diapause periods, and can provide valuable information that can influence pest-management strategies (Kiritani 1963, Toscano and Stern 1980, Herbert and Toews 2011, Herbert and Toews 2012).

We report sampling data from two patches of kudzu in South Carolina during 2013, 2014, and 2015. The study included 1) every other week sweep-net sampling to describe the seasonal abundance of adults and nymphs of *M. cribraria,* 2) every other week

sampling of kudzu vines to enumerate egg masses and eggs per mass, and 3) dissections of collected female adults to evaluate reproductive status.

Materials and Methods

Sampling. Sampling was conducted in two patches of kudzu located 25-km apart, near Blackville, SC (patch A: latitude 33.300919ºN, longitude -81.317369ºW; 1.3 ha and patch B: latitude 33.51342ºN, longitude -81.25637ºW; 1.4 ha), in 2013, 2014, and 2015. In 2013, twenty sweeps (two sets of ten sweeps) were performed every other week within the main vegetation from 2 May to 22 August in patch A (nine sampling weeks) and from 9 May to 14 August in patch B (eight sampling weeks). In 2014, fifty sweeps (five sets of ten sweeps) were performed biweekly within the main vegetation from 23 May to 26 September in patch A (ten sampling weeks) and from 29 May to 2 September in patch B (eight sampling weeks). In 2015, fifty sweeps (five sets of ten sweeps) were performed every other week within the main vegetation from 11 March to 22 October in patch A (16 sampling weeks) and from 3 March to 3 August in patch B (11 sampling weeks). Each set of sweeps was collected in plastic bags (30 x 50 cm) and frozen (-20°C) in the laboratory before enumeration of adults (2013), males and females (2014 and 2015), small nymphs (first, second, and third instar; 2013, 2014, 2015), and large nymphs (fourth and fifth instar; 2013, 2014, 2015). Counts were standardized per ten sweeps. One bag of ten sweeps was lost on 27 July 2015 from patch A. The five random sets of ten sweeps on 22 April and 6 May 2015 (patch A) and 5 May 2015 (patch B) were mistakenly pooled into one sample bag, hence, mean standard errors could not be calculated for these dates.

In addition to sweep-net sampling, random samples of 25 (2013) or 10 (2014 and 2015), 1-m kudzu vines were clipped, bagged and returned to the laboratory, where vines were examined for hatched and unhatched egg masses. Hatched egg masses were classified as those that had at least one egg hatched within the mass. Unhatched egg masses had no hatched eggs within the mass. Eggs in unhatched egg masses (2013) or eggs in all masses (2014 and 2015) were counted. Egg masses were not sampled in July 2013. Observations of the white entomopathogenic fungus, *Beauveria bassiana* (Balsamo)*, attacking *M. cribraria* were noted in 2014 and 2015.

Dissections. In 2014, up to forty females collected from sweep-net sampling were dissected from each sampled patch. In 2015, ten collected females were dissected from each sampled patch. Female reproductive organs were evaluated and given a rating of 'non-reproductive', 'intermediate' or 'reproductive' based on their developmental stage, a classification previously used to study the population structure of the brown stink bug, *Euschistus servus* (Say) (Herbert and Toews 2011). Non-reproductive females had no visible eggs present in their ovariole chambers, and, hence, were either newly emerged or had already laid eggs. Intermediate females had ovaries that contained immature eggs with soft choria that were malleable when pressed with forceps. Reproductive females had mature eggs with hardened choria that filled the entire abdominal cavity.

Data Analysis. Adult, nymph, and egg data were expressed as untransformed means ± SEM. Temperature data were obtained and plotted (NOAA 2016) for each sampling week of 2015. As the number of female samples available to dissect per location differed over the course of the season, reproductive ratings (non-reproductive,

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intermediate, and reproductive) were calculated as proportions observed per week. The Cochran-Mantel-Haenszel test was used to compare the proportion of each reproductive rating across the season (JMP 2013).

Results

2013. An average of 13.6 ± 0.3 (patch A; $n = 153$) and 14.1 ± 0.5 (patch B; $n = 71$) unhatched eggs per mass were recovered. In total, 468 and 153 (patch A) and 257 and 71 (patch B) hatched and unhatched egg masses, respectively, were collected across all sampling dates. The maximum number of eggs recorded in one egg mass was 64 and the minimum recorded was one. In both patches, on the initial sampling dates (2 May and 9 May), over six unhatched egg masses per 1 m vine were documented, which decreased over time (Fig. 4.1A and 4.1B). Hatched eggs decreased over time in patch A only. Unhatched and hatched egg masses were collected on all sampling dates in both patches until the final sampling date 22 August (patch A) and 14 August (patch B).

In patch A, the first small nymphs were recorded on 30 May and reached a peak of 376 ± 200 per ten sweeps two weeks later (Fig. 4.2A). Small nymphs were present on every subsequent sampling date, with decreasing numbers over time. Large nymphs were first recorded two weeks after the first small nymphs were found and reached a peak of 584 ± 332 six weeks later (Fig. 4.2B). Large nymphs were still at high numbers (333 ± 71 per ten sweeps) when sampling stopped on 22 August. In patch B, the first small nymphs were recorded on 6 June and reached a peak of 124 ± 39 ten weeks later (Fig. 4.3A). Small nymphs were present on every sampling date. Large nymphs were first recorded two weeks

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after the first detection of small nymphs. Numbers increased until the last sampling date (14 August) when 308 ± 28 large nymphs per ten sweeps were recovered (Fig. 4.3B).

Adults were present in high numbers in both patches on the first sampling dates in May (Fig. 4.2C and 4.3C). Numbers initially decreased until the beginning of June, when numbers increased dramatically. Sampling on the last date in patch A and patch B recorded an average of 235 ± 54 (Fig. 4.2C) and 278 ± 133 (Fig. 4.3C) adults per ten sweeps, respectively.

2014. In patch A, eggs per mass averaged 12.7 ± 0.8 ($n = 29$ hatched, 28 unhatched). The number of eggs per mass reached a maximum of 27 and a minimum of four. In patch B, eggs per egg mass averaged 11.2 ± 0.9 ($n = 13$ hatched, 7 unhatched), with a maximum of 17 and minimum of two eggs per egg mass. Unhatched egg masses were observed in both patches on the first sampling dates in May (Fig. 4.1C and 4.1D). In patch A, the number of unhatched egg masses per 1 m vine decreased gradually until none were recovered on 29 July. Hatched egg masses increased, peaking at approximately 1.5 egg masses per 1 m vine on 1 July, and declined until none were observed on 12 August (Fig. 4.1C). Hatched egg masses were present on the first sampling date in patch B (Fig. 4.1D). Egg masses decreased during the course of the season until no unhatched (23 July) and hatched (5 August) masses were observed.

Small and large nymphs were recovered on the first sampling date in patch A (Fig. 4.2A and 4.2B). Small nymphs reached a peak of 96 ± 17 per ten sweeps on 1 July, two weeks before the peak of large nymphs at 227 ± 33 per ten sweeps. No nymphs were recorded on the last sampling date (26 September). In patch B, small nymphs were present

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on 29 May, and large nymphs were collected two weeks later for the first time (Fig. 4.3A and 4.3B). Peaks of small (30 ± 13 per ten sweeps) and large nymphs (58 ± 13 per ten sweeps) coincided on 5 August before decreasing to approximately zero at the beginning of September.

In patch A, over ten sampling dates, 1120 females and 943 males were collected. The sex ratio was biased towards females ($\chi^2 = 15.186$; df = 1; $P < 0.0001$). In patch B, over eight sampling dates, 624 females and 558 males were collected. The sex ratio did not differ significantly from a 1:1 ratio ($\chi^2 = 3.685$; df = 1; $P = 0.0549$). Adults were present, but in low numbers on the first May sampling dates (patch A, 2.2 ± 1.4 per ten sweeps; patch B, 19 ± 8 per ten sweeps). Numbers gradually rose to a peak of 156 ± 31 per ten sweeps on 29 July (patch A) and 113 ± 16 per ten sweeps on 5 August. Numbers decreased gradually, until the last sampling date when 1 ± 0.7 (patch A) and 5 ± 2 (patch B) adults per ten sweeps were recorded. On 12 August and subsequent weeks *B. bassiana* was observed in patch A (Fig. 4.2).

2015. New kudzu stems and shoots starting emerging from the first week of April in both patches (Fig. 4.2 and 4.3). In patch A, over ten sampling dates ($n = 100$ vines), 12 hatched and 64 unhatched egg masses were collected. The average number of eggs per egg mass was 16.1 ± 0.9 (range 5-32). Collections of hatched egg mass were infrequent through the course of the season and never exceeded an average of 0.5 per 1 m vine (Fig. 4.1E). Hatched egg masses remained constant through June and July (around 1 egg mass per 1 m vine) before decreasing. No egg masses were collected after 11 August. In patch B, over eight sampling dates ($n = 80$ vines), only four hatched egg masses and six unhatched egg

masses were collected (average egg per mass, 13.57 ± 4.1). The first egg mass was collected on 27 May, and the last egg mass was collected on 22 July (data not shown).

Small nymphs were collected at the beginning of June in both patches, and large nymphs were collected two weeks later (Fig. 4.3A and 4.B). In patch A, 598 females and 575 and males were collected over 16 sampling dates. The adult sex ratio did not differ from 1:1 ($\chi^2 = 0.451$; df = 1; $P = 0.5019$). Adults were first recovered as temperatures began to rise on 22 April, and were present throughout the spring and summer, with a peak of 67 ± 23 on 1 July, two weeks after a peak of small instars 40 ± 19. Large instars showed two distinct peaks, on 1 July (20 ± 15) and 27 August (34 ± 13). Adult numbers per ten sweeps declined as temperatures declined, although *B. bassiana* was also observed in high prevalence on 16 September and 22 October (Fig 4.3).

In patch B, over 11 weeks, 69 females and 64 males were collected. The sex ratio did not differ from an expected 1:1 ratio ($\chi^2 = 0.188$; df = 1; $P = 0.6646$). Numbers of adults and nymphs were very low throughout the whole season. Adults were first collected during the first week of May, as temperatures began to increase. In patch B, adults reached a peak of 10 ± 3 on 7 July. Nymphs collected did not exceed four per ten sweeps for the entire sampling period.

Reproductive Status. In 2014, totals of 200 and 180 females were dissected from patch A and patch B, respectively. In 2015, 110 and 41 females were dissected from patch A and patch B, respectively. Females of all three reproductive stages were present in samples from patch A and patch B samples during both 2014 (Fig. 4.4A and 4.4B) and 2015 (Fig. 4.4C and Fig. 4.4D). A well-defined pattern of change in reproductive status

was observed in 2014 (patch A and patch B) and 2015 (patch A only) with a gradual reduction in the proportion of reproductive females (2014 patch A: $\chi^2 = 83.79$, df = 1, $P < 0.0001$; 2014 patch B: $\chi^2 = 8.90$, df = 1, $P < 0.0029$; 2015 patch A; $\chi^2 = 46.92$, df = 1, $P < 0.0001$) and an increase in the proportion of non-reproductive females collected during the course of the sampling period (2014 patch A: $\chi^2 = 78.32$, df = 1, $P < 0.0001$; 2014 patch B: $\chi^2 = 1133.68$, df = 1, $P < 0.0001$; 2015 patch A; $\chi^2 = 58.88$, df = 1, $P < 0.0001$). Week also influenced the proportion of intermediate females sampled in 2014 (2014 patch A: $\chi^2 = 5.91$, df = 1, $P = 0.015$; 2014 patch B: $\chi^2 = 12.70$, df = 1, $P = 0.0004$) but not in 2015 (patch A; $\chi^2 = 3.57$, df = 1, $P = 0.0589$; patch B; $\chi^2 = 1.4628$, df = 1, $P = 0.3365$). No pattern across week was identified in patch B in 2015 (Fig. 4.4D; reproductive; $\chi^2 = 2.188$, df = 1, $P = 0.1390$, intermediate; $\chi^2 = 1.4628$, df = 1, $P = 0.2265$; non-reproductive; $\chi^2 = 0.59$, df = 1, $P = 0.4405$).

Discussion

Kudzu is one of the primary developmental hosts of *M. cribraria,* alongside soybean (Zhang et al. 2012, Medal et al. 2013, Blount et al. 2015). Life stages and female population structure of *M. cribraria*, as assessed through reproductive status, were documented and described from two patches of kudzu in South Carolina. We observed oviposition on vine stems and tips in groups of parallel rows or singly, as noted by Zhang et al. (2012). Average egg numbers within a mass were similar to data reported in 2010 and 2011 from Georgia collections (Zhang et al. 2012) but we observed lower egg masses per 1 m kudzu vine in both South Carolina patches, in all years. Zhang et al. (2012)

described two ovipositional periods in April and late June or July. While early season peaks of egg masses were observed in South Carolina (Fig. 4.1), the absence of a second oviposition period in 2013 can be attributed to the lack of sampling for eggs in July, though a peak of nymphs and adults late in summer was reported. Consistent low densities of egg masses, adults, and nymphs likely explain the lack of clear oviposition peaks in 2014 and 2015. Unhatched egg masses were collected into August, as in previous studies (Zhang et al. 2012) and corresponded with intermediate and reproductive females sampled into August in both 2014 (Fig. 4.4A and 4.4B) and 2015 (Fig. 4.4B). Overlapping generations, occupying the same location, are not uncommon in long-lived, multivoltine hemipterans (Kiritani 1963, Toscano and Stern 1980). Adult dispersal from kudzu has not been fully characterized and could be a factor in lack of distinct generation differentiation. Assessments of dispersal capacity of overwintered, first generation, and second generation populations relative to reproductive status and kudzu are required. With the addition of an understanding of dispersal cues, the threat to soybean in an area surrounding kudzu could be assessed.

Population dynamics of *M. cribraria* illustrated by biweekly sweep-net sampling in kudzu in South Carolina mirrored general patterns observed by weekly sampling from individual kudzu vines in Georgia (Zhang et al. 2012). As in Zhang et al. (2012), the generation occurring on kudzu at the beginning of spring was not the result of reproduction in kudzu, in the same year, as no nymphs were present before the first adult was collected (Figs. 4.2 and 4.3), confirming dispersal of overwintered adults. Overwintered females generally entered kudzu as reproductive or intermediate females; however, non-

reproductive females were collected in May 2015 before nymphs were observed (Fig. 4.4B and 4.4D), suggesting that some individuals may remain in reproductive diapause for longer periods of time. Varying egg development times have also been reported in the pentatomid *E. conspersus* Uhler in California (Toscano and Stern 1980). Alternatively, females may have oviposited on another host elsewhere and dispersed into our kudzu sampling locations. Patterns of emergence in the spring have not been well characterized for *M. cribraria*; however, due to early sampling in 2015, we reported an association between increasing temperature and colonization of kudzu (Figs. 4.2C and 4.3C). Colonization of kudzu in April suggested that *M. cribraria* likely emerge from overwintering sites at that time. Other available plants might provide an alternative 'first' host for the overwintered generation. Late-season collections in 2015 determined that adults disperse from kudzu into presumed overwintering in a state of reproductive immaturity, an observation consistent with other hemipterans who overwinter as adults (Kiritani 1963, Toscano and Stern 1980, Herbert and Toews 2011, Herbert and Toews 2012). Golec and Hu (2015) addressed overwintering biology of *M. cribraria* with respect to reproductive development in females and males and reported egg formation was not observed in mated and virgin females collected from overwintering sites until the end of February, which is also consistent with our observations. Stimuli that induce and terminate diapause in insects include photoperiod and temperature (Denlinger 2002). Studies to determine the role of these factors for *M. cribraria* will provide valuable information to help with predictions of peak density, and, hence, awareness of overwintering ecology could assist with management strategies for *M. cribraria* in soybean.

We observed a reduction in mean number of adults and nymphs of *M. cribraria* collected per ten sweeps from 2013-2015 (Figs. 4.2 and 4.3). A decrease in *M. cribraria* populations has also been observed in other parts of the southeastern US (Musser et al. 2016). In 2013, in North Carolina, 25.4% of soybean hectares were infested with *M. cribraria*, of which 12.3% were above the economic threshold (Musser et al. 2014). A year later, although a similar number of hectares were infested, only 3% of these were above economic threshold (Musser et al. 2015). In 2015, only 3.6% of soybean hectares were infested, of which 0.2% were above the economic threshold (Musser et al. 2016). The plataspid was first reported in South Carolina, and more specifically Blackville, Barnwell County (where this study was conducted), in 2010. This area experienced lower and more extreme temperatures in January and February in 2014 and 2015 than in years previous (Table 4.1). Little is known about the behavior and ecology of overwintering *M. cribraria*, but overwintering mortality and patterns of spring emergence of other hemipteran pests have been well documented in soybean in South Carolina (Jones and Sullivan 1981). Extreme temperatures, equivalent to those observed in 2014 and 2015, resulted in 100% mortality of overwintering populations of the southern green stink bug, *Nezara viridula* (L.). This resulted in low populations of the economically important polyphagous pest in subsequent years (Jones and Sullivan 1981). Natural enemies of *M. cribraria* include the egg parasitoid, *Paratelenomus saccharalis* (Dodd) (Hymenoptera: Platygastridae) (Gardner et al. 2013), the adult tachinid fly parasitoids *Strongygaster triangulifera* (Loew) and *Phasia robertsonii* (Townsend) (Golec et al. 2013, Ruberson et al. 2013), the entomopathogenic fungus *B. bassiana* (Seiter et al. 2014) and a mermithid nematode

(Stubbins et al. 2015). Indeed, *B. bassiana* was observed attacking *M. cribraria* in patch A in 2014 and 2015, and a mermithid nematode was found in adults (2014 and 2015) and nymphs (2015) collected from a nearby soybean field (Stubbins et al. 2015, F.L.S., unpublished data). Although we did not observe other natural enemies attacking or infecting *M. cribraria*, the covert nature of these egg and adult parasites suggests that their presence could easily be overlooked. It is likely that a combination of abiotic and biotic factors contributed to population reduction from 2013-2015. Future work evaluating tolerances of *M. cribraria* to extreme temperatures and impact of natural enemies would provide empirical evidence to assess these hypotheses.

We investigated the patterns of seasonal abundance of *M. cribraria*, during three separate time periods, in two patches of kudzu in South Carolina. Population structure for females of *M. cribraria* over two time periods was also reported for the first time. As reproductively mature adults emerge from overwintering sites in April as temperatures begin to rise, the vast distribution and coordinated bud emergence of kudzu ensures the invasive weed is colonized until October. Monitoring populations of *M. cribraria* in kudzu and combining seasonal patterns with those in soybean may assist growers, county Extension agents, consultants, and farm managers to predict the severity of the pest in late-planted soybean.

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Table 4.1. Air temperatures and number of subfreezing nights during January and February in Blackville, SC during 2011-2015[a].

| Month | Year | Temperature (°C) | | | | Number of subfreezing nights |
		Average maximum	Average minimum	Overall average	Extreme minimum	
January	2011	11.1	-0.7	5.2	-8.4	20
	2012	16.7	2.9	9.8	-7.9	10
	2013[b]	17.7	5	11.3	-3.2	6
	2014[b]	10.9	-1.6	4.6	-11.7	19
	2015[b]	7.1	1.3	7.0	-9.4	12
February	2011	17.3	4.4	10.9	-3.8	7
	2012	17.8	4.6	11.2	-7.9	4
	2013[b]	14.6	2.5	8.2	-4.9	8
	2014[b]	Incomplete data-set				
	2015[b]	6.2	-0.2	5.9	-10.5	14

[a] Blackville, SC, climate observation station (latitude 33.35 °, longitude -81.33 °, elevation 96.6m; 6km and 20km away from patch A and patch B, respectively) developed by the National Oceanic and Atmospheric Administration (NOAA) [b] Years kudzu sampling was carried out in this study.
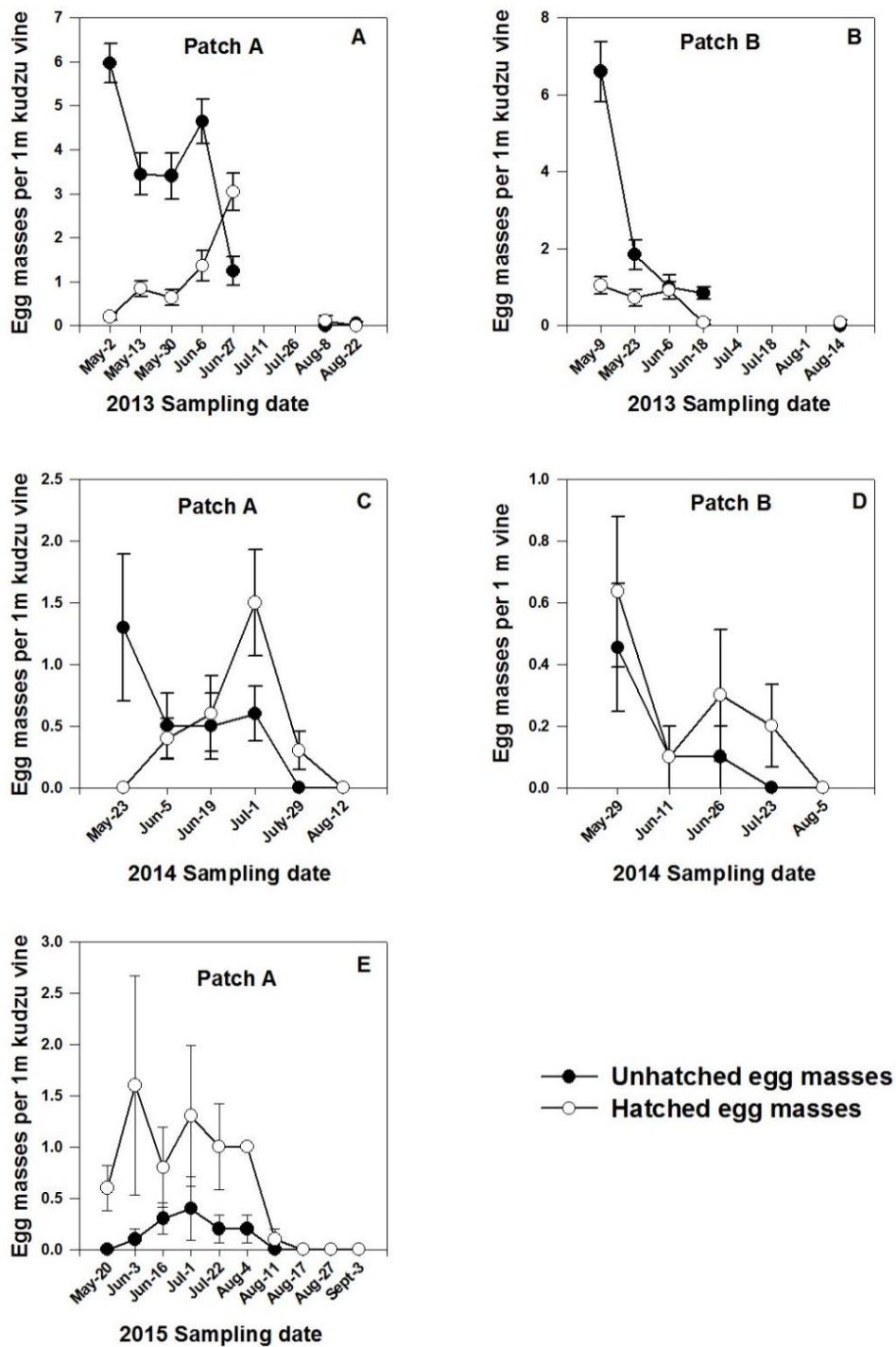
Fig. 4.1. Mean number of hatched and unhatched egg masses of *Megacopta cribraria* (± SEM) per 1-m kudzu vine in (A) patch A, 2013; (B) patch B, 2013; (C) patch A, 2014; (D) patch B, 2014; and (E) patch A, 2015, near Blackville, SC.
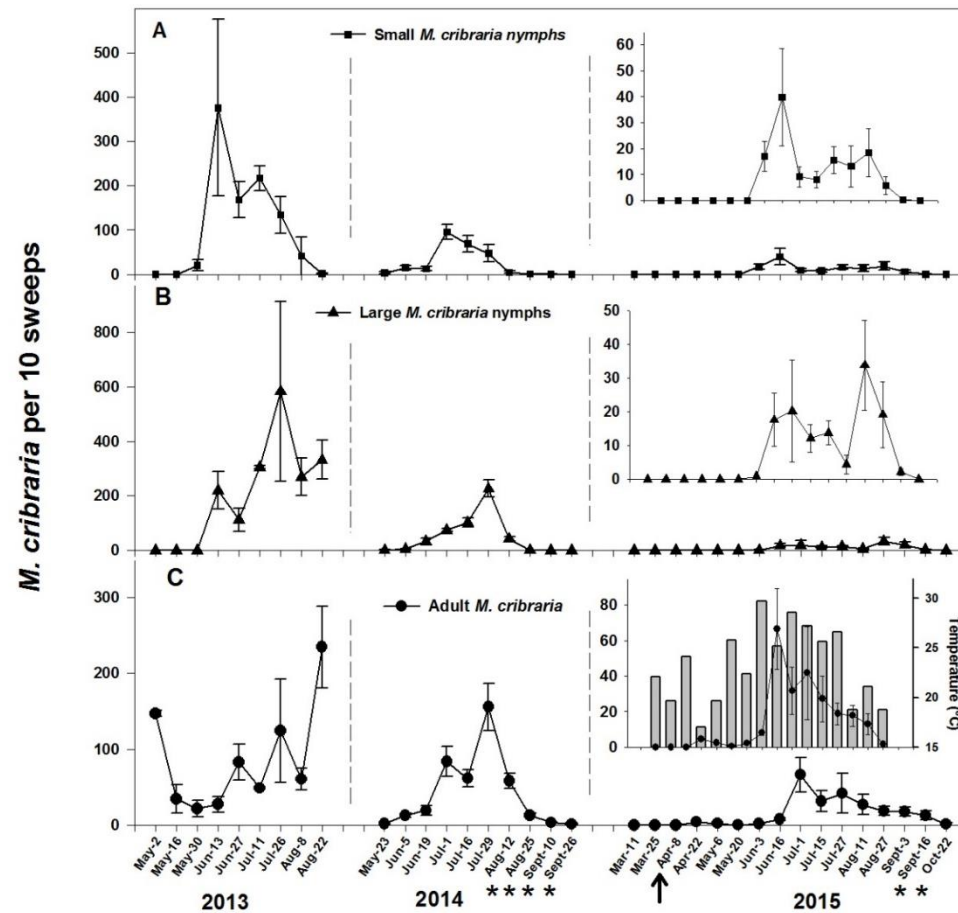
Fig. 4.2. Densities (± SEM) of (A) small nymphs, (B) large nymphs, and (C) adults of *Megacopta cribraria* in patch A of kudzu near Blackville, SC during 2013, 2014, and 2015. Asterisks indicate observations of *Beauveria bassiana*. Arrow indicates initiation of kudzu vegetative growth. All insets represent 2015 densities corresponding to the main chart but with a more appropriate scale, highlighting reduced densities of *M. cribraria*. Bars represent average temperature recorded at respective sampling dates.
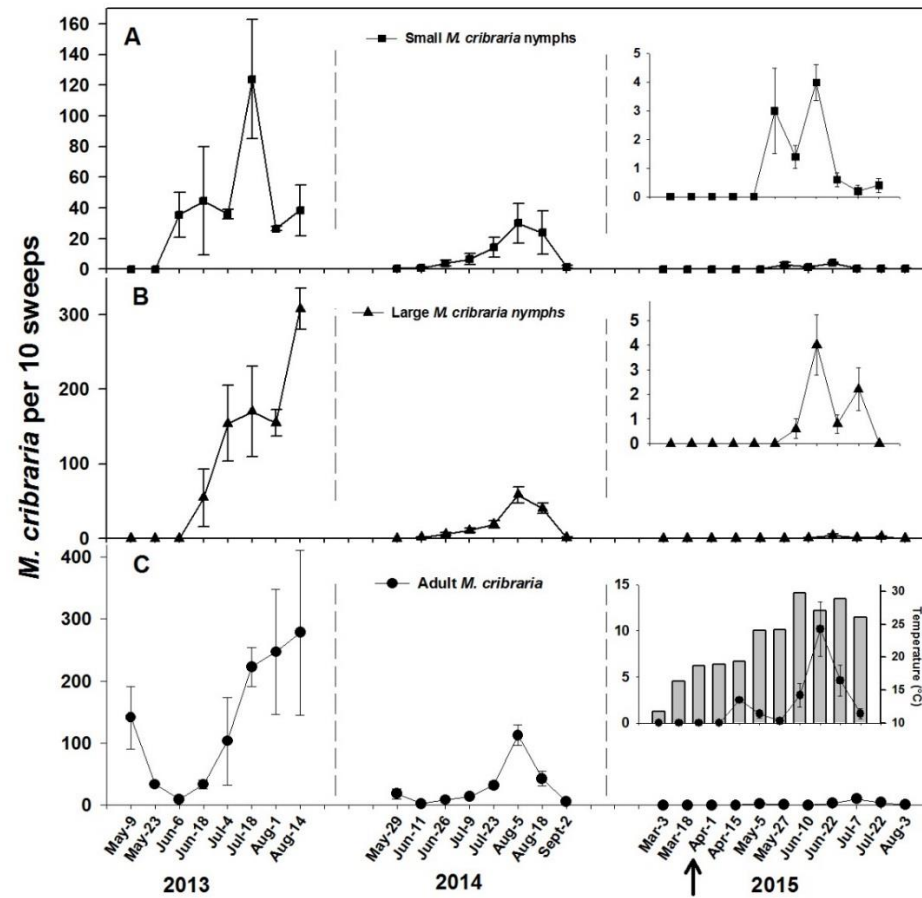
Fig. 4.3. Densities (± SEM) of (A) small nymphs (B) large nymphs, and (C) adults of *Megacopta cribraria* in patch B of kudzu near Blackville, SC, during 2013, 2014, and 2015. Arrow indicates initiation of kudzu vegetative growth. All insets represent 2015 densities corresponding to the main chart but with a more appropriate scale, highlighting reduced densities of *M. cribraria*. Bars represent average temperature recorded at respective sampling dates.
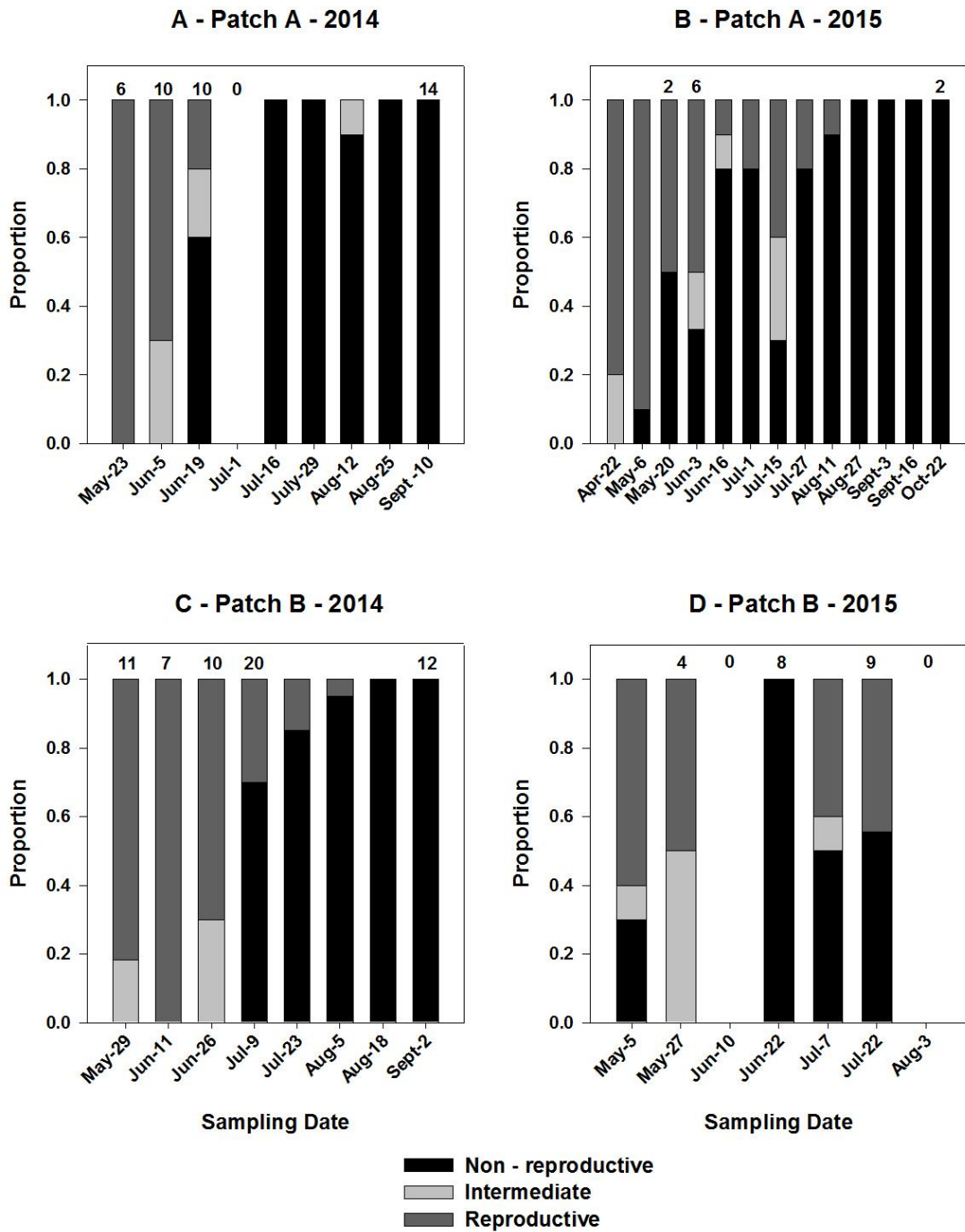
Fig. 4.4. Average proportion of non-reproductive, intermediate, and fully reproductive female *Megacopta cribraria* present per week sampled from (A) patch A in 2014; (B) patch A in 2015; (C) patch A in 2015; and (D) patch B in 2015. Numbers above bars represent the number of females dissected, when 40 (2014) and 10 (2015) were not available at each sampling date.

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CHAPTER V

STYLET PENETRATION BEHAVIORS OF THE INVASIVE *Megacopta cribraria*

(HEMIPTERA: PLATASPIDAE) USING ELECTROPENETROGRAPHY, WITH

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A native to Asia and the Indian subcontinent, the phytophagous kudzu bug, *Megacopta cribraria* (Fabricius) (Hemiptera: Plataspidae) was first sighted congregating on buildings in northeast Georgia in October 2009 (Ruberson et al. 2013). It has since spread through thirteen states and the District of Columbia (Gardner 2016). In addition to feeding on kudzu, *Puereria montana* (Loureiro) Merrill variety *lobata* (Willdenow), and some other legumes, the insect also feeds on soybean, *Glycine max* (L.) Merrill, and has become an established pest of the crop throughout the southeastern US (Zhang et al. 2012, Seiter et al. 2013).

In the southeastern US, *M. cribraria* completes two generations per year, and both can develop directly on soybean, with no need for an alternate host (Del Pozo-Valdivia and Reisig 2013). Adults and nymphs use piercing-sucking mouthparts to feed on soybean vegetative structures, but, unlike closely related pentatomids, they do not feed on seed or pods (Del Pozo-Valdivia and Reisig 2013, Seiter et al. 2013). Feeding-induced stress and injury can result in lowered soybean yield (Seiter et al. 2013). From 2011 to 2014, over $6.5 million of soybean yield losses plus insecticide treatment costs have been attributed to *M. cribraria* in the southern US, which is an underestimation of costs, as Georgia and South Carolina did not factor into these surveys (Musser et al. 2012, 2013, 2014, 2015).

The economic impact is expected to increase if populations continue to spread into additional soybean growing regions in the US (Zhu et al. 2012).

In the seven years since discovery, *M. cribraria* research has primarily aimed at managing this newly established pest in soybean, including investigating biological control prospects (Ruberson et al. 2013), insecticide efficacies and timings (Seiter et al. 2015a, Seiter et al. 2015b) and cultural practices (Blount et al. 2016, Del Pozo-Valdivia et al. 2016), but many important characteristics of the biology and ecology of the pest remain unknown. For example, despite the economic importance of this pest, few aspects of its feeding behavior have been studied. A reliable tool for understanding and monitoring insect feeding behaviors and mechanisms is electropenetrography (EPG), where an insect and plant are wired into an electrical circuit (McLean and Kinsey 1964). Stylet insertion into the plant closes the circuit and activities, such as ingestion, are converted into records of voltage change called waveforms. The measured voltage change is obtained from the resistance change produced from the varying conductivity of the saliva, phloem sap, and water that circulate through the mouthparts. Acquired waveforms can be identified, described, quantified, and statistically analyzed.

With a focus on the hemipteran suborders Auchenorrhyncha (e.g., leafhoppers) (Almeida and Backus 2004, Stafford and Walker 2009) and Sternorrhyncha (e.g., aphids, whiteflies, and psyllids) (Janssen et al. 1989, Symmes et al. 2008, Bonani et al. 2010), it took over a quarter of a century from EPG development (McLean and Kinsey 1964) for the first study on Heteroptera to be performed on the coreid *Anasa tristus* DeGeer (Bonjour et al. 1991). Since then, only ten studies, (eight additional species from three heteropteran

families), have been carried out, compared with over 200 studies with Sternorrhyncha and Auchenorrhyncha insects. These include the mirids *Lygus hesperus* Knight (Cline and Backus 2002, Backus et al. 2007), *Trygonotylus caelestialium* (Kirkaldy) (Suzuki and Hori 2014), and *Stenotus rubrovittatus* (Matsumura) (Suzuki and Hori 2014), the coreid *A. tristus* (Cook and Neal 1999, Maskey 2010), the blissids, *Blissus insularis* Barber and *B. occiduus* Barber (Rangasamy et al. 2015), and the pentatomids *Edessa meditabunda* (Fabricius) (Lucini and Panizzi 2016), *Piezodorus guildinii* (Westwood) (Lucini et al. 2016), and *Nezara viridula* (L.) (Cooke 2014). Valuable quantitative and qualitative information has been obtained on the feeding behaviors of these heteropteran agricultural pests, such as probing frequency and duration on both host and non-host plants. Coupling EPG data with histological (Backus et al. 2005), videomicrography (Cline and Backus 2002, Joost et al. 2006), and artificial diet (Cline and Backus 2002, Joost et al. 2006) studies enables waveform correlation with specific activities, such as probing behaviors while exploring plant surfaces or salivation events inside plant tissues.

Studies using EPG have been applied to answer a vast number of research questions, ranging from the effect of assorted plant cultivars (Serrano et al. 2000, Garzo et al. 2002, Alvarez et al. 2006, Rangasamy et al. 2015) or insecticide treatments (Joost and Riley 2005, Serikawa et al. 2012) on feeding behavior to investigations of plant-pathogen transmission (Fereres and Collar 2001, Almeida and Backus 2004, Backus et al. 2009). To our knowledge, there are no published EPG studies regarding the family Plataspidae. Similarly, there are no studies regarding the gross and finer aspects of mouthpart morphology for *M. cribraria* or relationships between body size and rostrum length

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through nymphal development. Morphological studies such as these can complement EPG findings (Anderson et al. 2006) or be used to highlight and identify feeding and life history constraints (Hochuli 2001).

The objectives of this study were (1) to provide the first documentation and characterization of *M. cribraria* waveforms, (2) to determine the biological meaning of waveforms through histological means, (3) to document the mouthpart morphology of adults and nymphs of *M. cribraria*, and (4) determine the relationship between rostrum length and body size of nymphs through development. This information will provide a basis for future comparative studies for this invasive species and supplement research aimed at optimization of integrated management strategies.

Materials and Methods

Plants and Insects. Four soybean seeds (variety AG6931, maturity group VI) were planted in square plastic pots (100mm x 100mm 120mm), thinned to two plants per pot on emergence, and allowed to grow under greenhouse conditions at the Clemson University Edisto Research and Education Center (EREC) in Blackville, SC. Plants with three to four unrolled trifoliate leaves (V3/4) were used as the feeding substrate for the duration of the study. Overwintered males and females of *M. cribraria* were collected from *Wisteria* spp. at EREC by shaking branches into sweep nets. After separation from debris, adults were caged on soybean plants (planted as above) in greenhouse conditions. Adults were allowed to mate, and emergent nymphs were transferred onto fresh plants in separate cages. On nymph final molt, adults were transferred to smaller cages containing a soybean plant and

placed in a chamber at 25 ± 2 °C with 16:8 (L:D) photoperiod and 65-95% relative humidity.

Insect wiring. Female adults (7-21 days old) were removed from the soybean food source, placed in individual plastic medical specimen cups fitted with mesh lids, and starved for 8 hours prior to wiring. A 2-cm copper wire was soldered to a brass nail. Gold wire (diameter = 0.05mm, length = 2cm, Alfa Aesar, Ward Hill, MA) was wrapped around the copper wire and fixed with silver conductive paint (Ladd Research Industries, Burlington, VT). A loop (approximately 800µm diameter) was made at the hanging end of the gold wire using fine forceps. To ensure minimal insect damage during the tethering process, sticky tape was prepared by rubbing it against clothing to collect lint. The tape was then firmly fastened (sticky side upwards) to a petri dish with additional tape. Starved insects were temporarily immobilized by placing their venter on the lint covered tape. Silver conductive paint was applied to the exposed insect pronotum with a toothpick and allowed to dry for 20s. The gold wire loop was dipped in silver conductive paint until covered and applied directly to the silver paint on the pronotum. Once the paint had dried, the insect was removed from the tape with soft-touch forceps. The insects were allowed to hang for approximately 15 minutes to acclimate to the wire before being tested.

EPG Equipment and Data Acquisition. A four-channel AC-DC monitor (Backus and Bennett 2009) was used to monitor feeding of *M. cribraria* female adults on soybean plants. To reduce external static and non-static electric fields, plants, wired insects, head-stage amplifiers (held by clamps on metal tripods), and Plexiglas stages (80mm x 150mm), for immobilization of plant stems (held by a 'helping hand' system of alligator clips and a

cast iron base), were placed in a Faraday cage (61cm x 61cm x 56cm) constructed of hardware cloth (19 gauge). A stem was laid down on the Plexiglas stage and fixed with strips of Parafilm (Bemis Flexible Packaging, Neenah, WI). A 24-gauge copper wire (3cm in length) was inserted into the soil to serve as the plant electrode providing current to the plant. The brass nail, attached to the insect by copper and gold wires, was placed in the head stage amplifier. A DI-720-USB Data Acquisition Logger (Dataq Instruments, Akron, Ohio) digitized the data output associated with stylet probing at 100 samples per second. Windaq Lite software (Dataq Instruments, Akron, Ohio) was used to acquire and store data. Two insects were monitored concurrently. All recordings were made with AC applied signal at a substrate voltage of 75mV and amplifier gain of 45%. The head stage amplifier was set at an input resistance of $10^7\Omega$. Insects were recorded for nine hours under laboratory conditions throughout the night.

Waveform Analysis. We followed the waveform terminology as in Almeida and Backus (2004). Briefly, waveform phases were characterized from recordings at Windaq compression 24-35, waveform families at a Windaq compression of 5-10, and types at Windaq compression 2-5. Identified *M. cribraria* waveforms were described qualitatively and quantitatively. Relative amplitude (defined as the position of a waveform corresponding to the baseline voltage relative to the highest peak voltage), absolute amplitude (defined as the actual size of the waveform from the lowest valley to the highest peak, expressed as a relative percentage), and repetition rate were estimated by selecting three random time points within a particular phase/family/type, for every usable recording.

Z1 and Z2 waveform types (i.e. walking, grooming, holding still) were observed frequently and often for long periods of time. Therefore, to approximate duration percentages for waveform type, each was confirmed for presence or absence every 20min from the start of the recording (n=27 per 9 h recording). All other waveform measurements were calculated precisely (Backus et al. 2007). These measurements included NWEPI (defined as the number of waveform events per probe per insect), WDI (defined as the duration sum of all events of a certain waveform made by an individual insect), and WDPI (defined as the duration average of all events of a certain waveform per probe per insect).

Histological Studies and Analysis. Pentatomomorpha, in addition to making watery saliva, produce rapidly hardening, gelling saliva that surrounds the stylets as they penetrate tissue (Miles 1972). Location of salivary sheath tissue can be exploited to correlate waveforms with biological activity. A second set of female adults were recorded at all times of the day using the methods described above. When an identifiable waveform was observed on the Windaq Lite Software, stylet penetration was artificially terminated by pulling the insect from the soybean stem. The approximate point of stylet insertion was marked with a pen. A 1-cm piece of stem surrounding the stylet insertion mark was fixed in formaldehyde-acetic acid-alcohol for histological processing by the Histology Core Facility, Department of Animal and Veterinary Sciences, Clemson University. In brief, samples were run in an enclosed tissue processor (Leica ASP300S, Leica Microsystems, Bannockburn, IL) in a series of ethanol solutions of increasing concentrations (70-100%). Xylene was used as a clearing agent to displace the ethanol, and each sample was infiltrated with paraffin wax at 58ºC. Paraffin blocks were serially thick-sectioned at 10µm with a

rotary microtome. Ribbons were mounted on slides and stained with Safranin O (Sigma Aldrich, St. Louis, MO) and counterstained with Fast Green FCF (Sigma Aldrich, St. Louis, MO). Salivary sheaths were examined with an Olympus BH2 light microscope (Olympus Optical, Tokyo, Japan), with objectives 4x and 10x, and images were obtained with ProgRes®CapturePro (v.2.8.8, Optical Systems, Jena, Germany).

Scaling Relationships and Mouthpart Morphology. Adults and nymphs were collected from a patch of kudzu (latitude 33.300919º, longitude -81.317369º; 1.5ha) near Blackville, SC, approximately 12 km from EREC using sweep-net sampling on 17 June 2015 and preserved in 80% ethanol. Images of males (n=10), females (n=10), and nymphs (n=10 per instar) were captured with a Nikon E600 compound microscope (Nikon Corporation, Tokyo, Japan) and QCapture software (QImaging, Burnaby BC, Canada) and calibrated to a 1-mm scale. Adult ventral length, nymph dorsal body length, and adult/nymph pronotum width were measured along the maximal dimension of each; inter-ocular width was measured between the base of each compound eye; and rostrum length was measured from the top of the labrum to the apex of labium segment IV. All image analyses were performed in ImageJ v1.47 (National Institutes of Health, Bethesda, MD). Exported data were analyzed with ANOVA and means separated with Student's t-test ($\alpha =$ 0.05) or Tukey's honest significance test (JMP 2013). Measured values (mm) were log transformed, and the influence of indicators of body size (independent variables: body length, pronotum width, and inter-ocular width) on the dependent variable, rostrum length, were estimated by linear regression (JMP 2013). An isometric relationship occurs when body size is proportional to other body parts (slope = 1), and an allometric relationship

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exists when body size varies disproportionately to other body parts (slope ≠ 1) (Stern and Emlen 1999).

Three sample types of female adults, fifth instars, and first instars (collected as above) were prepared for scanning electron microscopy: (1) whole head (female adults) or whole insect (fifth and first instars); (2) labium; and (3) stylet bundle by chemical drying with 100% hexamethyldisilazane (Sigma Aldrich, St. Louis, MO) for twenty minutes. After air drying for 48h, samples were mounted on double-sided conductive carbon tape affixed to individual aluminum stubs (26mm) and sputter coated with gold-palladium for two minutes at a controlled pressure of 80 millitorr (~5 nm thickness, Hummer® 6.2 Sputtering System, Anatech Ltd., Union City, CA). Individual samples were examined and imaged in a Hitachi S-3400N scanning electron microscope with a 5-kV accelerating voltage (Hitachi High-Technologies, Krefeld, Germany).

Results

EPG waveform characterization

General Overview. Only females responsive for nine hours (n=14, total number of probes=18) were included in analyses. Of these females, four were still probing when the recordings stopped, and so these were eliminated from some phase/family quantitative analyses. In one instance, a female performed four distinguishable probes within 9h, but due to suboptimal wiring, phases within the probe could not be distinguished, so this was removed from 'within probe' analyses. Thus, the minimum sample size was nine. Three distinct waveform phases (designated 'non-probing', 'pathway', and 'ingestion') were

observed and described for *M. cribraria* on soybean stems (Table 5.1). Non-probing (family Z) waveforms were composed of Z1, Z2, and Z3 types. Females performed probing (defined as stylet insertion to stylet removal) behaviors in a consistent, stereotypical manner (Fig. 5.1). Probing waveforms were composed of pathway (family G and family H) and ingestion phases (family I). Stylet insertion always followed Z3, followed by G, H, and I families. Family H was observed between ingestion phases on one occasion. Probes always culminated with G, followed by a sharp voltage spike, representing stylet removal.

From a total access time of 5,400 minutes, ten insects spent 1,447 minutes in the probe phase, representing 27% of the total time females had access to the plant. Probing duration per insect ranged from 31-285 min (mean 144 ± 74min [SD]). Probing duration per probe ranged from 31-225 min (117 ± 59min). Most females (86%) followed a trend of one probe per 9h (1.3 ± 0.8, range 1-4). Time elapsed until first probe was extremely variable, with a range of over 8.5h (mean 5.02 ± 2.93h). The number of probes and the number of waveform events per probe per insect were consistent, resulting in similar WDI and WDPI values (Table 5.2). The percentage of recording time spent in each phase (PRT) was highly skewed, with the non-probing phase, and more specifically Z2 (non-moving), being the longest. Similarly, the percentage of time spent in each phase during probing (pathway and ingestion) was highly skewed, with ingestion representing over 90% of the total probing time (Table 5.2).

Non-probing Phase: (Z1, Z2, and Z3). Waveform types Z1, Z2, and Z3 occurred when the stylet was not inserted in the soybean stem and represented approximately 75% of all identified waveforms (Table 5.2, Fig 5.1A and B). Z1 was characterized by an

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irregular set of jagged waveforms, often with high relative and absolute amplitude, that was easily correlated through visual observations with walking and grooming (Fig. 5.1A, Table 5.1). Z1 was observed directly before Z3 and subsequent stylet insertion (see below) in 67% of probes (n=18), with a mean duration of 80 ± 122 s (range 14–459s). After stylet removal (probe termination), Z1 was observed in 93% of cases with a mean duration of 188 ± 162 s (range 0.42-500s). As the baseline, Z2 had very little variation in voltage and occurred when the insect was stationary (Fig. 5.1A, Table 5.1). This behavior was observed for the majority of the non-probing phase (Table 5.2). Z3 was always observed before stylet insertion (see below) and consisted of up to five (2.72 ± 1.07, Fig 5.1B) regular, rounded waves, with 20-25% relative amplitude and a low absolute amplitude.

Pathway Phase (Family G and Family H). Immediately following Z3, the voltage spiked (100% relative amplitude), representing stylet insertion into the soybean tissue and the start of a true probe. Family G was always observed twice in a single probe. At the start, G had a recognizable pattern consisting of an average of nine 'blocks' (mean 3.79 s ± 0.97 per block) consisting of a spiked peak followed by lower-amplitude spiked waves, with a frequency of 5 Hz (Table 5.1, Fig. 5.1B). Block voltage decreased, steeply, at a rate of 9.3% per second (Fig 5.1B). Family G observed at the end of the probe had a similar but reversed pattern, resulting in an increase in voltage, but with a significantly shorter duration ($F = 101.5$; df = 1, 18; $P < 0.0001$). Family H consisted of low absolute amplitude irregular waveform patterns, frequently interspersed with longer, wider spikes with a higher absolute amplitude (Fig 5.1C). Family H always followed initial G, but was also seen at the end of a probe, in nine out of ten instances, before the stylet was removed from soybean tissues

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and between ingestion phases (1/10). There was no significant difference between durations of H at the start of the probe compared with at the end of the probe ($F = 1.28$; df $= 1, 28$; $P = 0.2718$)

Ingestion (Family I). The ingestion phase (Fig. 5.1B) followed immediately after H and consisted of a uniform frequency waveform with low absolute amplitude and consistent relative amplitude. The waveform started off as a sinusoidal monophasic wave with rounded peaks but transitioned into different forms, consisting of biphasic periods (Fig 5.1D and E). Wave frequency (4Hz) remained constant throughout the ingestion phase, regardless of transitional form.

Correlation Between Ingestion Waveform and Stylet Sheath Position

Stylet sheaths terminated in the vascular tissue ($n=5$). Family H was associated with termination in the non-conductive phloem bundle cap fiber cells (n=1; Fig. 5.2A). When females were removed during the ingestion phases, three of four sheaths terminated in phloem (Fig. 5.2B); one of these branched twice before reaching the phloem (Fig 5.2C). One sheath branched to the pith before terminating in the xylem (Fig. 5.2D).

Mouthpart Morphology and Scaling Relationships

Rostrum length, body length, pronotum width, and inter-ocular width increased through nymph development (Table 5.3). Each body size parameter (body length, pronotum width, and inter-ocular width) increased with nymphal instar. The majority of growth occurred between the fourth and fifth instar. Linear regression analysis indicated a significant relationship between rostrum length and body length ($F = 870.74$; df = 1, 48; $P < 0.001$; $r^2 = 0.95$) and pronotum width ($F = 1249.78$; df = 1, 48; $P < 0.001$; $r^2 = 0.96$).

Both relationships showed negative allometry (body length, slope = 0.66; F = 222.05; df = 1,48; P < 0.001, pronotum width, slope = 0.63; F = 435.09; df = 1,48; P < 0.001). There was a significant (F = 660.95, df = 1, 48, P < 0.001, r^2 = 0.93) isometric (slope = 1.03, F = 0.61; df = 1; 48; P = 0.440) relationship between rostrum length and inter-ocular width (Fig. 5.4). Although males and females had significantly different body lengths and pronotum widths, inter-ocular widths and rostrum lengths were similar (Table 5.4).

The mouthparts of *M. cribraria* are similar to those of other piercing-sucking insect pests and include the labrum, labium, and stylet bundle of mandibular and maxillary stylets (Fig. 5.3). Overall, there were no detectable structural morphological differences through development or sex. The four-segmented labium lies between the coxae and is bisected by the stylet groove, in which lies a stylet bundle formed from the mandibular and maxillary stylets (Fig. 5.3A and B). A short tear-drop shaped labrum overlies the labial groove of the first labial segment (Fig. 5.3A). The mandibular stylet tips, externally convex and internally concave, are pointed and grooved with irregular prominences (Fig. 5.3C and D). The maxillary stylets are encased by the mandibular stylets (Fig. 5.3C). The pointed tips curve inwards and appear to interlock with each other (Fig. 5.3C and E). The labium apex contains multiple sensilla (Fig. 5.3F). On each side of the stylet groove opening, a group of ten bilaterally symmetric sensilla were observed. The sensilla were peg-like in shape, with a uniform diameter and a blunt apex. Longer, more pointed hair-like sensilla, surrounded the peg-like sensilla and were more concentrated and numerous in the fifth instar and adult stage. Finally, tuft like projections were observed in a row at the center of the labial tip between peg-like sensilla in adults and fifth instars.

Discussion

The threat of invasive heteropteran pests to US agriculture persists (Panizzi 2015), and significant economic losses associated with their establishment underlines the need for robust research regarding their feeding biology. At the time of invasion, there is often insufficient biological information concerning the invasive species due to literature that is frequently printed in the language of the invader's native country (Bansal et al. 2013) or because the insect is regarded as a non-pest in previously established distributions; both these scenarios applied to the invasive *M. cribraria* (Eger et al. 2010). Furthermore, in contrast to defoliators, with feeding behavior that is easy to observe, manipulate, and research (Scriber and Slansky 1981), feeding research on invasive piercing-sucking pests presents complications because of the hidden injury caused by the covert nature of stylets inserted into plants. Hence, *M. cribraria* stem-feeding behavior has been previously limited to intermittent observations (Seiter et al. 2013). The EPG technique allows research to go beyond superficial and restrictive observation, permitting detailed analyses of core feeding behaviors (McLean and Kinsey 1964). We collected high resolution waveform data and corresponding histological data to investigate stylet penetration behaviors of *M. cribraria*. Our study presents only the fourth published recording of a pentatomomorphan heteropteran species and the first from the family Plataspidae.

Female adults performed a set of stereotypical sequential behaviors immediately prior to and during a probe (Fig 5.1A), as observed in other heteropterans (Backus et al. 2013, Lucini and Panizzi 2016). Immediately prior to stylet insertion and the start of the probe, Z3 can be interpreted as presumed labial dabbing on the stem surface (Cline and

Backus 2002). The first identifiable probing waveform (pathway phase; G) resembled those describing the pathway phase in the blissids *B. insularis* and *B. occiduus* (G and H) (Backus et al. 2013), the pentatomids *E. meditabunda* (Em1) (Lucini and Panizzi 2016), *P. guildinii* (Family P) (Lucini et al. 2016), and *N. viridula* (Cooke 2014), as well as auchennorrhyncans (Almeida and Backus 2004, Backus et al. 2005, Miranda et al. 2009) and sternorrhynchans (Prado and Tjallingii 1994, Bonani et al. 2010). Although we did not correlate waveform G with a stylet terminus in soybean tissue, there is evidence from EPG correlation studies with other species to suggest that pathway waveforms represent penetration and salivary sheath formation as the stylet advances through the plant tissue (Joost et al. 2006, Bonani et al. 2010, Lucini and Panizzi 2016, Lucini et al. 2016). Interestingly, *M. cribraria* waveform H (pathway phase), associated with phloem bundle cap fiber cells, resembles waveform D produced by the Asian citrus psyllid, *Diaphorina citri* Kuwayama described in Bonani et al. (2010) and Serikawa et al. (2012). Waveform D correlated with salivary sheath termini in an unknown cell type in phloem tissue and was proposed to represent a transition from pathway to phloem phases as we observed in *M. cribraria.* Further histological work to ascertain correlations over a range of periods during both G and H family waveforms, together with division of pathway waveforms into constitutive types as in Almeida and Backus (2004), will be important for future inferences of *M. cribraria* feeding behavior.

The constant ingestion waveform has been observed in the majority of published EPG recordings, including those performed on non-hemipterans, such as the Thysanoptera (Kindt et al. 2006), and consistently correlate with ingestion from phloem (Bonani et al.

2010, Lucini and Panizzi 2016), xylem (Almeida and Backus 2004, Joost et al. 2006, Cooke 2014, Lucini et al. 2016), and mesophyll (Stafford and Walker 2009). An ingestion waveform frequency of around 4 Hz is also consistent across a number of insects (Bonani et al. 2010, Lucini and Panizzi 2016, Lucini et al. 2016). Fungi growth ("sooty mold") on honeydew secretions from *M. cribraria* on host plants suggests *M. cribraria* ingests from phloem. Reports from Malaysia (Maschwitz et al. 1987, Waldkircher et al. 2004), Sumatra (Waldkircher et al. 2004), Sri Lanka (Waldkircher et al. 2004), Borneo (Blüthgen et al. 2006) and Cameroon (Dejean et al. 2000, Gibernau and Dejean 2001), indicating trophobiotic mutualisms between honeydew secreting plataspids and ants, provide further evidence for phloem-feeding capabilities in Plataspidae. Our histological studies corroborate phloem feeding in *M. cribraria* (Fig. 5.2B and C), with the majority of salivary sheaths observed in association with the phloem during an ingestion waveform. We could not distinguish vascular tissue ingestion waveforms and we, therefore, suggest that *M. cribraria* is a phloem feeder that hydrates occasionally (Fig. 5.2D). Ingestion from both xylem and phloem has been observed in aphids (Tjallingii 2006), a stink bug (Lucini and Panizzi 2016), psyllids (Bonani et al. 2010), and chinch bugs (Backus et al. 2013), presumably as a strategy to maintain water balance (Spiller et al. 1990). Transitioning ingestion waveforms, as observed in this study, have been documented in *B. insularis* (J-I1 and JI-2) (Backus et al. 2013), *D. citri* (E1 and E2) (Bonani et al. 2010), and aphids (E1 and E2) (Tjallingii 2006). These transitions have been assigned separate proposed activities consisting of, for example, salivation into a phloem sieve element followed by salivation

during sieve element ingestion (Tjallingii 2006). Further work is necessary to assign these activities in *M. cribraria.*

Previous EPG studies have recognized a specific waveform, denoted as a J or X wave. Characterized by an abrupt transition and thought to be species-specific, the waveform is produced directly before stylet penetration inside a preferred target tissue (Wayadande and Nault 1993, Carpane et al. 2011, Backus et al. 2013, Lucini and Panizzi 2016). We did not observe X or J waves in any recordings; however, Backus et al. (2013) acknowledged that both appearance and presence/absence of a waveform could be altered by different input impedances and applied voltages. Hence, multiple selections of input impedances may have revealed previously undetectable waveforms. An ultimate but time intensive goal for any comprehensive understanding of an insect's feeding behavior is to assemble a thorough waveform library using different input impedances, voltages, and direct vs. alternating currents, to ensure all possible activities are perceived and to enable differentiation between xylem- and phloem- ingestion waveforms.

Over-representation of non-probing activities, a high percentage of 'rest' (Z2), and large variations in waveform event duration between individuals, despite controlling for many factors, are familiar attributes associated with heteropteran EPG recordings (Cline and Backus 2002, Backus et al. 2007, Lucini and Panizzi 2016). Inactivity (Z2) can be conceivably explained as periods of food assimilation or energy preservation. Time-lapse video coupled with EPG recordings correlated the Z2 waveform with *L. hesperus* antennae held out in a raised position, suggesting that non-movement might also be a period for volatile detection (Cline and Backus 2002, Backus et al. 2007). In our study, the increase

in activity directly before stylet penetration and directly after stylet removal was similar to reports in *L. hesperus* (Backus et al. 2007), and suggested a period of surface exploration before an attempt to feed, followed by walking away or grooming after ingestion occurred.

Feeding duration and frequency are amongst the many factors that determine crop damage and, thus, potential yield loss. Stems were the focus of this study, as *M. cribraria* are most often seen feeding on these structures in soybean in the US (Seiter et al. 2013). Females of *M. cribraria* perform long bouts of probing, consistent with those durations (from EPG studies and visual observations) of closely related pentatomids (Simmons and Yeargan 1988, Cooke 2014, Lucini and Panizzi 2016), but it will be important for future EPG studies with *M. cribraria* to consider differences in feeding behavior between sexes and nymphal instars. Adult comparisons were performed on heteropterans using EPG, and differences in feeding activity in two species of rice ear bugs, *T. caelestialium* and *S. rubrovittatus*, when mated or unmated and between sexes were observed (Suzuki and Hori 2014). No heteropteran EPG studies have compared behaviors between nymphal instars. Historically, salivary sheath, salivary flange counts, and periodic visual observations have been used to estimate feeding activity indirectly, with differences reported between instars (Panizzi et al. 1995, Depieri and Panizzi 2011, Zeilinger et al. 2015). Our results provide a baseline to investigate the feeding differences between life stages and how changes in food consumption may associate with reproductive status.

Temporal patterns of behavior in plataspids have not been well characterized, although mating (in aggregated clusters) has been reported from the afternoon until early morning of the next day (Hosokawa and Suzuki 2001). Furthermore, few reports exist as

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to how environmental factors impact *M. cribraria* feeding behavior. EPG (Suzuki and Hori 2014) and the similar, but less versatile, electronically monitored insect feeding (EMIF) system (Shearer and Jones 1996, Wiman et al. 2014) have been employed to assess heteropteran probing activities over 24 h, with differing temporal activity reported in the species examined. Knowledge of temporal feeding patterns coupled with the effect of abiotic factors can be exploited to improve pest sampling and management strategies (Binns and Nyrop 1992, Krupke et al. 2006, Son et al. 2012, Wiman et al. 2014). We recorded females of *M. cribraria* for 9 h during the night to gather information for preliminary waveform characterization but had difficulties obtaining feeding data during the day (36% success rate, compared with 57% during the night), an observation also alluded to in Maskey (2010) while monitoring *A. tristis*. Therefore we propose that *M. cribraria* preferentially feed during scotophase or that activities in the laboratory may disturb normal daytime feeding behaviors.

Although adults and nymphs of *M. cribraria* are most often found on kudzu and soybean in the US, there is still uncertainty and contradiction as to which other legume species serve as developmental hosts, food sources, or merely alighting structures (Gardner et al. 2013, Medal et al. 2013, Blount et al. 2015, Golec et al. 2015). Furthermore, the role of non-leguminous plants remains ambiguous. Studies using EPG can be a screening tool for possible plant hosts, and it is especially applicable for insects that are recently established or those that are predicted to invade an area in the future (Cook and Neal 1999, Sandanayaka et al. 2007, Sandanayaka and Backus 2008, Sandanayaka and Page-Weir 2009). The ability of an insect to obtain nutrients from a non-developmental host, such as

A. tristis on cucumber, *Cucumis melo* L. (Cook and Neal 1999), gives increased opportunity to find a suitable host and, hence, has ecological and management implications. Qualitative and quantitative results from this study provide a foundation to test for predicted host plant species and to determine their ecological role in the life cycle of *M. cribraria*.

Short-term solutions such as effective, fast-acting insecticide applications are used to manage invasive insects until more sustainable, long-term strategies, such as resistant crop varieties, can be developed. Information regarding feeding behavior and biology is paramount to ensure development of these strategies. Indeed, from a historical perspective, host plant resistance to manage soybean pests has been extremely successful (Bansal et al. 2013). Monitoring insects using EPG has been used to study and characterize soybean resistance in phloem-feeding aphids (Diaz-Montano et al. 2007, Todd et al. 2016) and other agricultural pests. Our initial descriptive and quantitative results regarding waveform characterization at a cohort, insect, and probe level provide useful information and lay the groundwork for understanding treatment and resistance mechanisms in soybean varieties in the future.

The negative allometry between rostrum length and body length/pronotum width indicates that larger nymphs (and, therefore, later instars) have a proportionally smaller rostrum compared to body size, hence the relative growth of mouthparts changes disproportionately as body size increases. This implies that the rostrum acquires a disproportionately smaller allocation of resources during nymph development. These ontogenic changes in rostrum length may reflect changes in demands encountered by

nymphs. For example, fourth and fifth instars develop wing buds, necessary structures for adult dispersal. The allometry may also suggest a change in feeding behavior between nymphal instars and should be a point of study, as discussed previously.

We present the first known reports of *M. cribraria* mouthpart ultrastructure using scanning electron microscopy. Gross mouthpart morphology of *M. cribraria* is typical of other salivary-sheath producing hemipterans (Snodgrass 1935). Mouthparts of nymphs and adults showed a high degree of gross and fine morphological similarity. Mandibular stylet tips were similar in shape and texture to other phytophagous pentatomid (Depieri and Panizzi 2010), blissid (Anderson et al. 2006), and coreid (Rodrigues et al. 2007) herbivores. Comparable looking apical labial sensilla have been reported in other phytophagous hemipterans (Backus 1988, Leopold et al. 2003, Anderson et al. 2006, Parveen et al. 2015). Elucidation of dendritic innervation from a transmission electron microscope investigation in Aleyrodidae suggested a chemosensory or mechano-sensory function for apical sensilla (Walker and Gordh 1989). Although we did not establish this role in *M. cribraria*, we can hypothesize a similar function in the plataspid.

Data presented here have improved our knowledge regarding feeding and mouthpart morphology of *M. cribraria*. By providing details and insight into probing duration and frequency on soybean stems and confirming *M. cribraria* phloem-feeding behavior, future comparative and integrative studies of this important soybean pest have been made possible. Results provide a baseline for answering a number of basic but applied questions concerning *M. cribraria*, including identification of host plants, temporal feeding activity, and feeding behavior of other life stages, as well as providing valuable information

for screening of resistant soybean cultivars. Continued research on feeding behavior is crucial, if this pest is to be managed successfully and sustainably in the future.

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Table 5.1. Descriptions of six waveforms identified from AC-EPG recordings of fourteen females of *Megacopta cribraria* collected near Blackville, SC, placed on soybean stems at a voltage of 75 mV, input impedance of $10^7\Omega$, and 45% gain. Relative amplitude is defined as the approximate position of the waveform type in relation to the baseline voltage level and the highest voltage peak during the probe. Absolute amplitude is defined as the actual size of the waveform from the lowest valley to the highest peak, expressed as a relative percentage.

Phase	Family	Type	Relative amplitude (%)	Absolute amplitude (%)	Frequency (Hz)	Correlations	
						Histological studies	Putative activity
Non-probing	Z	Z1	0-100	-	Irregular	-	Walking, grooming[a]
		Z2	0	-	-	-	Standing still[a]
		Z3	20-25	6 (low)	0.5	-	Mouthpart dabbing[b]
Pathway	G		70-100	70 (high)	5	-	Mouthparts entering plant tissue, salivary sheath formation[b]
	H		25-35	8 (low)	Irregular	Phloem bundle cap fiber cells	Unknown, transition into and out of ingestion tissue
Ingestion	I		20-25	2-6 (low)	4	Phloem/xylem	Ingestion

[a] Based on visual observations [b] Based on other studies on phytophagous hemipterans

Table 5.2. Mean (± SD; ranges in parentheses) probe, insect, and event waveform level values used to study AC-EPG recordings of female *Megacopta cribraria* placed on soybean stems (n=9) at a voltage of 75 mV, input impedance of $10^7\Omega$, and 45% gain for nine hours.

Phase	Family	Type	NPW	NWEPI	WDI (min)	WDPI (min)	PRT (%)	PPT (%)
Non-probing	Z	Z1	9	-	-	-	16	-
		Z2	9	-	-	-	59	-
		Z3	9	1	0.15 ± 0.06 (0.09-0.24)	0.14 ± 0.05 (0.09-0.24)	< 0.001	-
Probing — Pathway	G (start)	-	9	1	0.61 ± 0.10 (0.46-0.78)	0.57 ± 0.11 (0.36–0.78)	0.08	0.4
	G (end)	-	9	1	0.11 ± 0.06 (0.05-0.24)	0.10 ± 0.04 (0.05-0.20)	0.02	0.1
	H	-	9	2.13 ± 0.33 (2-3)	12.57 ± 8.72 (3.04–25.48)	11.16 ± 7.28 (3.04–21.37)	2	9
Ingestion	I	-	9	1.11 ± 0.31 (1-2)	124.31 ± 74.87 (14.86-258.74)	109.94 ± 55.83 (14.86-202.21)	22.9	90.5

NPW, number of individuals that produced the specific waveforms; NWEPI, Number of waveform events per probe per insect; WDI, Waveform duration per insect; WDPI, Waveform duration per probe per insect; PRT, percentage of 9-h recording time; PPT, percentage of probing time

Table 5.3. Mean (± SE) body length, pronotum width, inner-ocular width, rostrum length for each *Megacopta cribraria* nymphal instar, collected from a patch of kudzu near Blackville, SC. Values in columns with a letter in common are not significantly different based on Tukey's honest significance test (α = 0.05).

Nymph instar	n	Body length[a] (mm)	Pronotum width[b] (mm)	Inter-ocular width[c] (mm)	Rostrum length[d] (mm)
1st	10	0.90 ± 0.02a	0.51 ± 0.02a	0.28 ± 0.01a	0.82 ± 0.004a
2nd	10	1.90 ± 0.02b	0.85 ± 0.06a	0.41 ± 0.02b	1.16 ± 0.06b
3rd	10	2.88 ± 0.06c	1.27 ± 0.10b	0.52 ± 0.03c	1.60 ± 0.07c
4th	10	3.90 ± 0.10d	2.23 ± 0.17c	0.68 ± 0.03d	2.04 ± 0.07d
5th	10	5.38 ± 0.06e	3.00 ± 0.04e	0.81 ± 0.03e	2.64 ± 0.03e

[a]$F = 828.45$, df 4, 45 $P < 0.0001$; [b]$F = 118.15$, 4, 45, $P < 0.0001$; [c]$F = 73.45$, df 4, 45 $P < 0.0001$, [d]$F = 176.06$, df = 4, 45 $P < 0.0001$

Table 5.4. Mean (± SEM, mm) body length, pronotum width, inner-eye width, rostrum length, and corresponding ratios for male and female adults of *Megacopta cribraria*, collected from a patch of kudzu near Blackville, SC. Values in columns with a letter in common are not significantly different based on Student's t-test ($\alpha = 0.05$).

Adult	n	Body length[a] (mm)	Pronotum width[b] (mm)	Inter-ocular width[c] (mm)	Rostrum length[d] (mm)
Female	10	5.11 ± 0.06a	4.03 ± 0.04a	0.88 ± 0.02a	2.54 ± 0.05a
Male	10	4.25 ± 0.05b	3.60 ± 0.03b	0.84 ± 0.02a	2.43 ± 0.05a

[a]$F = 124.55$, df 1, 19 $P < 0.0001$; [b]$F = 75.50$, 1, 19 , $P < 0.0001$; [c]$F = 2.92$, df 1, 19 $P = 0.1047$, [d]$F = 2.0198$, df = 1, 19 $P = 0.1724$
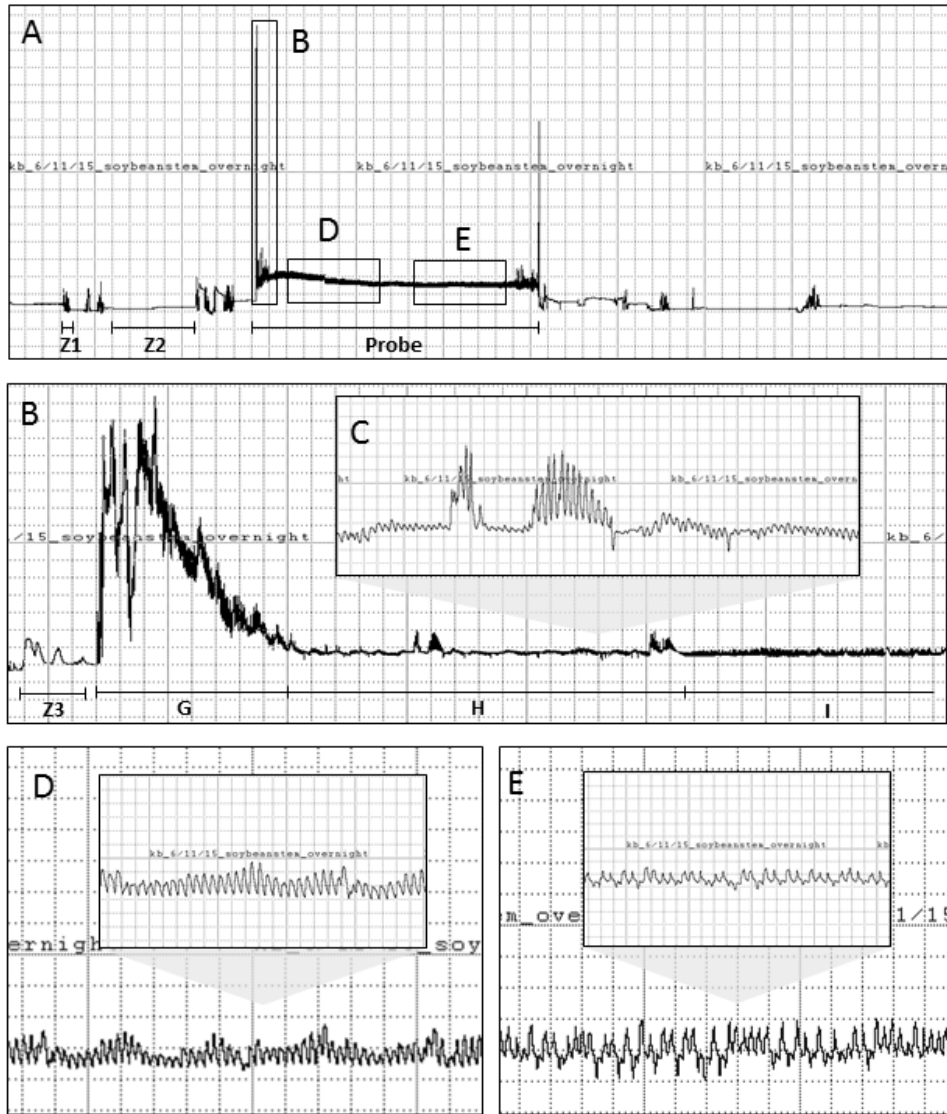
Fig. 5.1. Waveforms generated from a female *Megacopta cribraria* on an AC-DC EPG machine at an AC applied voltage of 75mV, input impedance of $10^7\Omega$, and 45% gain. (A) Compressed nine hour recording showing Z1, Z2 and probe (166 min; consisting of G, H, and I). Windaq compression 3062, 612.4 s/vertical division. (B) expanded view of box B in (A), representing the probe beginning with Z3 followed by G, H and then I. Windaq compression 10, 2 s/vertical division. (C) Expanded view of H waveform. Windaq compression 2, 0.4 s/vertical division. (D) Expanded view of box D in (A), ingestion (I) waveform. Windaq compression 10, 2 s/vertical division. Inset; expanded view of ingestion waveform. Windaq compression 2, 0.4 s/division. (E) Expanded view of box E in (A), ingestion (I) transitional waveform. Windaq compression 10, 2 s/vertical division. Inset; expanded view of ingestion transitional waveform. Windaq compression 2, 0.4 s/division.
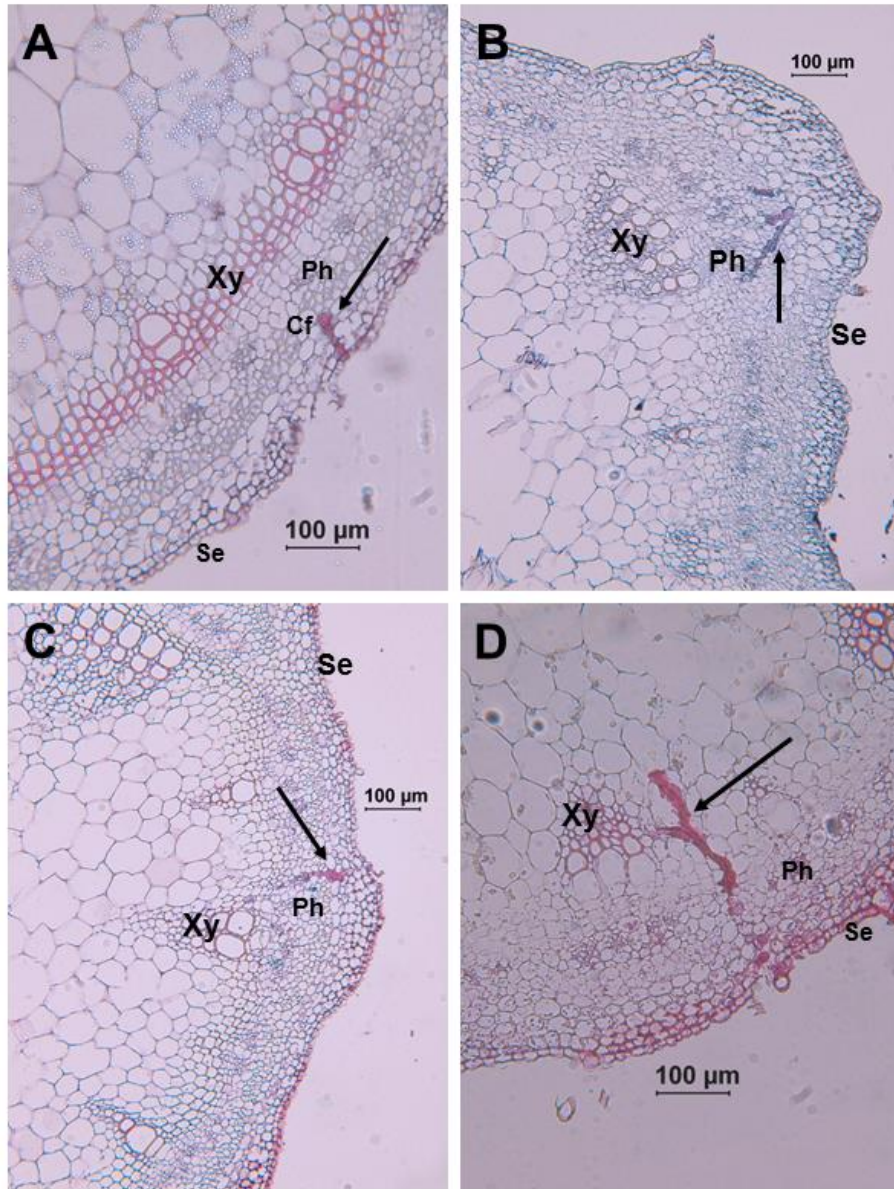
148

Fig. 5.2. Cross sections of soybean stem containing salivary sheaths (denoted by black arrows) from female *Megacopta cribraria*. (A) Salivary sheath ending in non-conductive phloem bundle cap fiber cells during recording of family H waveform (x20). Xylem (Xy); phloem (Ph); phloem bundle cap fiber cells (Cf); stem epidermis (Se) (B) Branched salivary sheath tip in phloem during recording of ingestion waveform (x10). Xylem (Xy); phloem (Ph); stem epidermis (Se) (C) Salivary sheath ending in in phloem during recording of ingestion waveform (x10). Xylem (Xy); phloem (Ph); stem epidermis (Se) (D) Branched salivary sheath tip in xylem during recording of ingestion waveform (x10). Xylem (Xy); phloem (Ph); stem epidermis (Se).
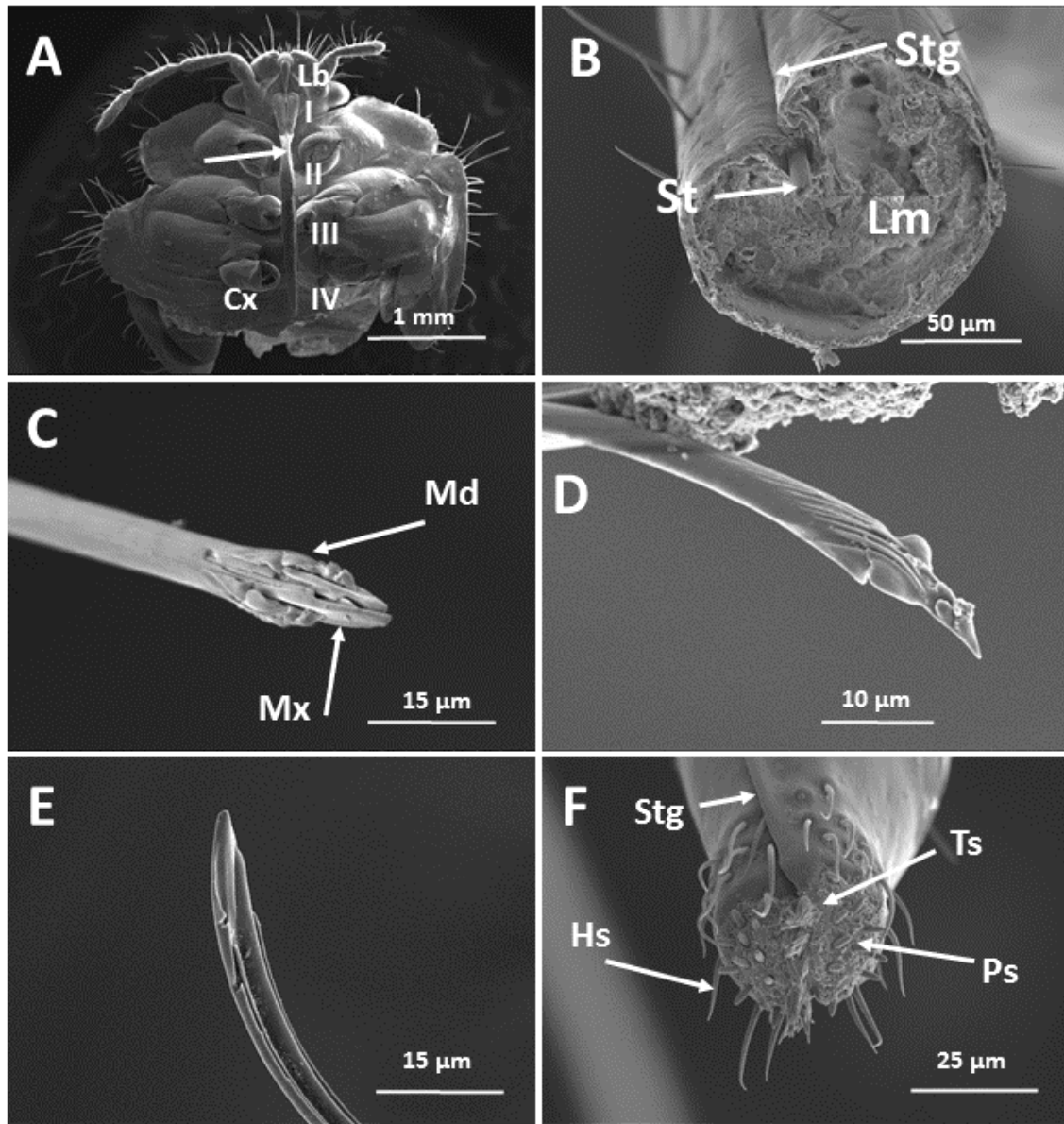
Fig. 5.3. Ultrastructure study of *Megacopta cribraria* mouthparts. Scanning electron microscopy images of (A) Ventral view of a fifth instar nymph. Labrum (Lb); first labial segment (I); second labial segment (II); third labial segment (III); fourth labial segment (IV); metathoracic coxa (Cx); arrow indicates exposed stylet. (B) Cross-section through the third labial segment of a female adult. Labium (Lm); stylet (St); stylet groove (Stg) (C) Distal mandibular (Md) and maxillary (Mx) stylets of a first instar nymph (D) Distal mandibular stylet details from a female adult (E) Distal maxillary stylet from a female adult (F) Apex of the fourth labial segment of a female adult. Hair-like sensilla (Hs); peg-like sensilla (Ps); tuft-like sensilla (Ts).
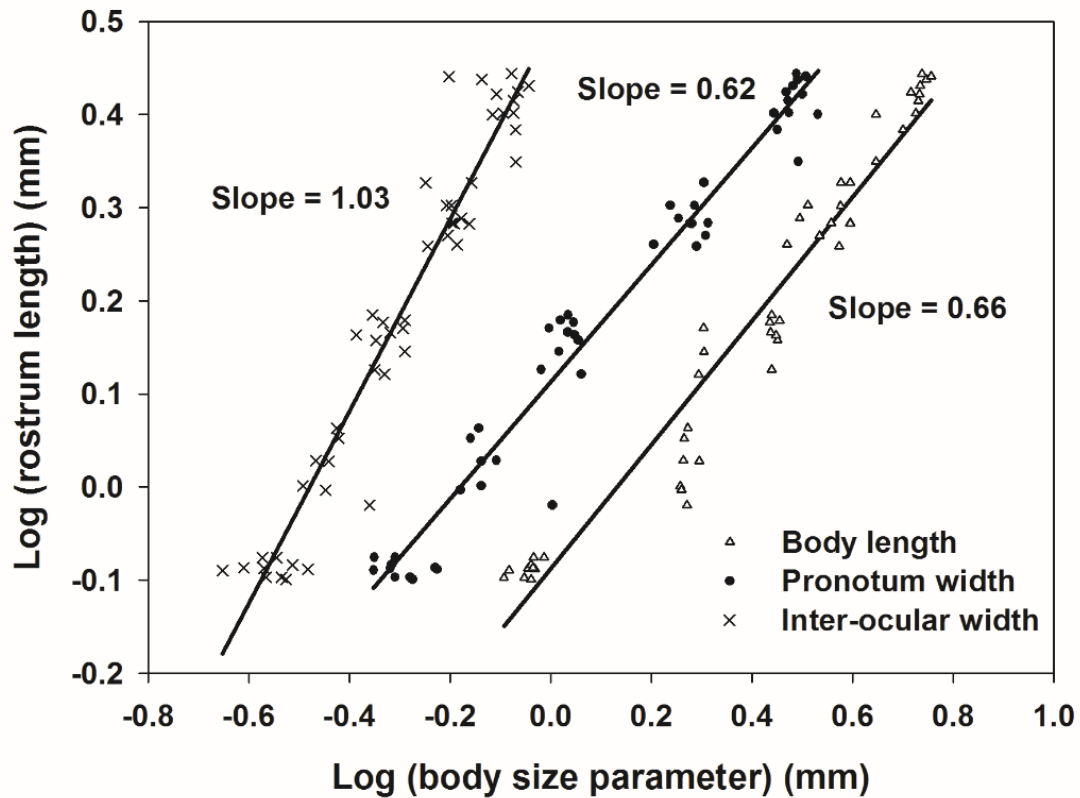
150

Fig. 5.4. Significant ontogenic negative allometry (slope < 1) of rostrum length against body length (F = 222.05; df = 1,48; $P < 0.001$) and pronotum length (F = 435.09; df = 1,48; $P < 0.001$) and the isometry (slope = 1) between rostrum length and inter-ocular width (F = 0.61; df = 1; 48; $P = 0.440$) for nymphs of *Megacopta cribraria*.

CHAPTER VI

FIRST REPORT OF A MERMITHID NEMATODE INFECTING THE INVASIVE

Megacopta cribraria (HEMIPTERA: PLATASPIDAE) IN THE UNITED STATES

Megacopta cribraria (Fabricius) (Hemiptera: Plataspidae), a native to Asia, was first discovered in Georgia, United States, in 2009 (Suiter et al. 2010). By 2014, the invasive plataspid had been reported in all of the southeastern states. The plataspid feeds on a wide range of leguminous plants, but its primary hosts are soybean, *Glycine max* (L.), and kudzu, *Pueraria montana* (Lour.) variety *lobata* (Willd.), in the United States (Zhang et al. 2012, Ruberson et al. 2013). In soybean, feeding by both adults and nymphs can affect overall crop yield (Seiter et al. 2013), establishing this insect as a serious soybean pest. Generalist, natural enemies of *M. cribraria* have been reported in the United States, including the predatory pentatomid *Euthyrhynchus floridus* (L), the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Seiter et al. 2014)*,* and the indigenous adult parasitoids *Strongygaster triangulifera* (Loew) (Diptera: Tachinidae) and *Phasia robertsonii* (Townsend) (Diptera: Tachinidae) (Golec et al. 2013, Ruberson et al. 2013). Furthermore, an egg parasitoid (Hymenoptera: Platygastridae) specific to *M. cribraria* and previously only found in the Old World*,* has been identified in field-collected egg masses in the southeastern United States (Gardner et al. 2013). Entomoparasitic nematodes have yet to be reported in *M. cribraria*. This study outlines the first of such nematodes to be found in both the invasive and endemic range of *M. cribraria*.

Materials and Methods

M. cribraria **Sampling, Nematode Collection and General Observations.** As part of an ongoing study to determine spatial patterns and to evaluate female reproductive development, *M. cribraria* adults were collected from Clemson University's Edisto Research and Education Center near Blackville, South Carolina, via sweep-net (38-cm diameter) sampling from five soybean fields (V8) ('Fields A-E'). Twenty sweeps across two rows (planted on 98-cm rows) at four distances from the field edge (0, 10, 20 and 40 m) on three transects were carried out per field and date (240 sweeps per field and date). Samples were collected in plastic bags (30 x 50-cm) and frozen (-20°C) in the laboratory before insects were counted. Females were summed across the three transects per field for each distance, and ten were partially dissected by removing the entire scutellum and pleural membrane to expose the internal organs (40 females per field). On 26 July 2014, nematodes were observed beneath the pleural membranes in the abdominal cavities of two females (single nematode per adult female). Nematodes were photographed with a zipScope 2M USB Digital Microscope (Aven Inc., MI) (Fig. 6.1), removed, and preserved in 80% ethanol. Sweep-net sampling and female dissections continued weekly in all five fields until the soybeans were too dry to sweep (R7; 29 September 2014). On 7 August 2014, 50 field-caught adult females and 50 field-caught adult males of *M. cribraria* were placed in a plastic rearing cage (30 x 30 x 30-cm; BugDorm, BioQuip) containing a soybean plant (R2) in order to observe parasitic juveniles exiting hosts and to potentially rear them to adults. The cage was placed in an environmental chamber (25°C, L:D 14:10, 70% humidity). Each day, dead adults were removed from the cage, placed in separate

containers with soil, and observed for nematode emergence. Emergent nematodes were stored in distilled water for preservation (Kaya and Stock 1997).

Nematode Identification. Examination of parasitic and post-parasitic juveniles under the microscope revealed well developed stichosomes, a diagnostic characteristic of the family Mermithidae (Kaya and Stock 1997). To further confirm this diagnosis, nematode DNA was extracted from three individuals using a Sigma Extract-N-Amp kit (XNAT2) (Sigma, St. Louis, MO). Sequences from two markers amplified using methods described in Vandergast and Roderick (2003) were used to identify the nematode. The 18S small ribosomal DNA was amplified with primers 18S-5F (5′GCGAAAGCATTTGCCAAGAA) and 18S-9R (5′GATCCTTCCGCAGGTTCACCT) and a portion of the mitochondrial COI gene was amplified with primers JB3 (5' TTTTTTGGGCATCCTGAGGTTTAT 3') and JB5 (5' AGCACCTAAACTTAAA AC ATAATGAAA 3'). The amplified products were loaded onto a 1.5% agarose gel and visualized using GelRed™ (Biotium). Magnetic beads were used to purify PCR products which were then sequenced in both directions with the ABI 3730 capillary sequencer (Applied Biosystems) in the DNA Laboratory (School of Life Sciences) at Arizona State University.

Results and Discussion

All nematode-infected females were collected from Field A (33.352723 N, -81.331518 W). In this field, out of 2564 females collected, 422 were dissected, with 20 of these parasitized by nematodes (4.7%). The level of infection reached a peak of 16% on 18

August, when the average number of females collected was 1.5 per 20 sweeps. The last recorded nematode infection rate was 2.5% on 7 September, when an average of 11 females were collected per 20 sweeps. Nematodes were always recorded in the hemocoel of the host abdomen with an attachment point in the thorax. Females of all reproductive status were infected, with 65% (n=13) containing fully developed eggs. Organ and tissue loss was observed in 85% (n=17) of females, suggesting that the majority of nematodes had been *in situ* for a prolonged period of time. Parasitic juveniles were found in isolation, though, on one occasion, two were observed within the hemocoel.

In the caging experiment, nematodes emerged from both males (n=7) and females (n=5) over the course of 14 days. All parasitic juveniles emerged from the caudal end of the adult, except one, which was observed to have a cephalad exit (Fig. 6.2).

A Basic Local Alignment Search Tool (BLAST) search through GenBank denoted that the amplified sequences had the closest match to the mermithid, *Agamermis spp.* COI gene. The top nine matches also matched mermithid sequences, supporting morphological identification. GenBank reference sequences are limited for this nematode group, but mermithids have been described infecting other species of closely related hemipteran families (Bhatnagar et al. 1985, Rahaman et al. 2000, Tarla et al. 2011), including field-caught pentatomids in the United States (Fuxa et al. 2000, Esquivel 2011, Kamminga et al. 2012). Further morphological and molecular studies are needed for complete identification of the mermithid to the level of genus and species.

Mermithid infection had also been documented in the brown stink bug, *Euschistus servus* (Say), collected from one pecan, *Carya illinoinensis* (Wangenh.) K. Koch, orchard

in Texas (Esquivel 2011), and in crop pests in India (Bhatnagar et al. 1985). Terrestrial mermithids live in the soil and can infect both immature and adult host insects directly from the soil or after short migration up a plant stem (Nickle 1981, Petersen 1985). Adult and nymph *M. cribraria* feed and develop on leguminous plant stems (Ruberson et al. 2013). The insect has a bivoltine life cycle where the second generation overwinters in leaf litter, behind tree bark, or in houses (Ruberson et al. 2013). We hypothesize that during leaf litter overwintering, juvenile mermithids entered adult plataspids or that during development on the soybean plant, any one of the stages were infected by a migrating mermithid. Host tissue damage was observed, but data to determine whether the mermithid reduced fecundity and life longevity or altered other areas of plataspid host physiology were not obtained.

Conclusion

We report infection of *M. cribraria* adults with a mermithid nematode. This discovery adds to the current inventory of native and non-native enemies currently attacking this invasive insect. It is unknown whether this nematode will have a significant role in reducing populations of *M. cribraria*. Mermithid nematodes have been reported to play an important role in the natural control of other hemipterans (Nickle 1981). Integrated pest management combines a range of complementary methods encompassing chemical, cultural, and biological approaches to prevent pest populations from reaching economically important levels. The economic importance of *M. cribraria*, both in rural and urban environments, underlines the need for combined management efforts in the United States. In the short term, effective insecticide applications are the most widely used, immediate

option exotic species control, but the continuous threat of insecticide resistance emphasizes the need to investigate other alternatives (Ruberson et al. 2013). Our observations and reports provide a basis for justified future research to examine the impact of mermithid nematodes on host fecundity and mortality of *M. cribraria* and to investigate their capacity to significantly reduce populations of the pest as a possible biological control option.

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Fig. 6.1. Parasitic juvenile nematode within abdomen of a female *Megacopta cribraria* collected from soybeans near Blackville, SC, during 2014.

Fig. 6.2. Post-parasitic juvenile nematode emerging from the cephalad end of a *Megacopta cribraria* male collected from soybeans near Blackville, SC, during 2014 and subsequently reared in an environmental chamber.

CHAPTER VII

Agamermis (NEMATODA: MERMITHIDAE) INFECTION IN SOUTH CAROLINA

AGRICULTURAL PESTS

Over 200 pentatomid species are present in North America (Froeschner 1988), of

which three, the green stink bug, *Chinavia hilaris* (Say), the brown stink bug, *Euschistus*

servus (Say), and the southern green stink bug, *Nezara viridula* (L.), are considered the

main agricultural pests of economic importance in the southeastern United States (Jones

and Sullivan 1982, Barbour et al. 1990, Greene et al. 2001). In 2014, stink bugs infested

2.5 million ha of cotton, *Gossypium hirsutum* L., (approximately half of the area planted

in the United States), destroying 135,000 bales and resulting in total damage estimated at

$106 million (Williams 2015). Damage was particularly severe in the southeastern United

States, representing the majority of total insect damage to the crop in the region (Williams

2015). Significant yield losses from the stink bug complex are also frequent in soybean,

Glycine max (L.) Merr. (McPherson and McPherson 2000). In 2014, stink bugs accounted

for 20% ($370 million) of the reported yield losses and management costs for insect pests

in the crop, in the southeastern United States (Musser et al. 2015). Recently, the invasion

and establishment of the highly polyphagous brown marmorated stink bug, *Halyomorpha*

halys Stål (Nielsen and Hamilton 2009), the redbanded stink bug, *Piezodorus guildinii*

(Westwood) (Temple et al. 2013a), and the kudzu bug, *Megacopta cribraria* (F.)

(Plataspidae; closely related to the Pentatomidae family) (Ruberson et al. 2013), have

added to the soybean pest complex. In Louisiana, *P. guildinii* was the most dominant stink

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bug species (54%) collected in soybean from 2008 to 2010 (Temple et al. 2013a). From 2011 to 2014, over $6.7 million in losses plus management costs were attributed to *M. cribraria* in the southern United States (Musser et al. 2012, 2013, 2014, 2015). Stink bugs can also be a pest in corn, *Zea mays* L. (Negrón and Riley 1987), peaches, *Prunus persica* L. (Rings 1957), sorghum, *Sorghum bicolor* L. (Young and Teetes 1977), tomato, *Solanum lycopersicum* L. (Michelbacher et al. 1952), and wheat, *Triticum aestivum* L. (Viator et al. 1983).

The widespread pest status of stink bugs across multiple crops in the United States, and particularly in the Southeast, often requires multiple applications of broad spectrum pyrethroid or organophosphate insecticides to preserve yields. Populations of stink bugs with decreased sensitivity to a broad range of insecticides have been reported in the United States (Baur et al. 2010, Temple et al. 2013b). Furthermore, insecticide usage can result in pest resurgence or secondary pest outbreaks through elimination of beneficial natural enemies (Ruberson et al. 1998). To reduce these threats, research has historically been conducted on integrating control tactics, including the identification and use of natural enemies as biological control. Indeed, a complex of parasitoids and predators, traversing phyla, are known to attack populations of both stink bugs (Jones 1988, Jones et al. 1996, Fuxa et al. 2000, Koppel et al. 2009, Esquivel 2011) and *M. cribraria* (Gardner et al. 2013, Golec et al. 2013, Greenstone et al. 2014, Ruberson et al. 2013, Stubbins et al. 2015) in the United States.

Terrestrial mermithids are a large group of obligate entomopathogenic nematodes that are considered important regulators for some insect populations, including hemipteran

pests (Kaburaki and Imamura 1932, Choo and Kaya 1990), because of their capacity to retard development, induce female sterility, and cause death on emergence (Kaiser 1991). Although characterized with a broad host range (Poinar 1979, Nickle 1981), research directed at this nematode group has often focused on observations in one host (Esquivel 2011, Tarla et al. 2012, Tarla et al. 2015). This study was prompted by observations of terrestrial mermithid nematodes in *M. cribraria* in South Carolina in 2014 (Stubbins et al. 2015) and designed to develop our knowledge regarding mermithid host range and prevalence. Specifically, we aimed to (i) determine prevalence of mermithid nematodes in economically important hemipteran pests in soybean in South Carolina, and (ii) identify mermithid nematodes using molecular tools.

Materials and Methods

Insect Sampling and Nematode Collection. A soybean (variety AG6934) field (33.352723 N, -81.331518 W) at the Clemson University Edisto Research and Education Center near Blackville, South Carolina [where mermithid nematode infection in *M. cribraria* had been previously documented (Stubbins et al., 2015)], was selected for sweep-net sampling in 2015. Twenty sweeps (38-cm diameter sweep net) across two rows (planted on 97-cm rows) at four distances from the field edge (0, 10, 20, and 40 m) on three transects (240 sweeps per date) were carried out weekly from 6 June (soybean stage R1; when *M. cribraria* adults were first observed in the field) until 9 October (soybean stage R7; 13 weeks). Samples were collected in plastic bags (30 x 50-cm) and frozen (-20°C) in the laboratory before enumeration of *M. cribraria* (from 6 June) and pentatomids (adults and

fourth/fifth instars; from 21 July). An adult sex ratio shift from an expected 1:1 ratio was analyzed using the chi square goodness-of-fit test. Collected *M. cribraria* (females from 6 June, males and fifth instars from 21 July) were summed across the three transects for each distance, and ten specimens (when available) of each life stage (up to 40 per field, per date) were partially dissected by removing the entire scutellum and pleural membrane (adults) or dorsum (nymphs) to expose the internal organs. Collected pentatomids were summed across field each week and the majority partially dissected (Table 7.1). Nematodes observed in the abdominal cavities of dissected insects were photographed with a zipScope 2M USB Digital Microscope (Aven Inc., MI), removed, and preserved in 80% ethanol. Nematode voucher specimens were deposited in the Clemson University Arthropod Collection (CUAC). On 30 July, due to high lepidopteran pest abundance, an application of indoxacarb (0.065 kg [AI] ha^{-1}, Steward EC, Dupont Inc., Wilmington, DE) was made.

Nematode Identification. Nematodes collected from *M. cribraria* (adult, $n = 3$; nymph, $n = 3$); *Acrididae* sp. (nymph, $n = 2$); *E. servus* (nymph, $n = 2$); other *Euschistus* sp. (adult, $n = 1$); *C. hilaris* (nymph, $n = 1$) were examined for morphological features (Olympus BH2 light microscope). Genomic DNA was extracted from individual parasitic juvenile nematodes [Table 7.1 (all $n = 1$); *M. cribraria* adult ($n = 1$); *M. cribraria* nymph ($n = 1$)] using Sigma Extract-N-Amp kits (XNAT2) (Sigma, St. Louis, MO). A portion of the 18S small subunit (SSU) ribosomal DNA (800bp) was amplified using the forward primer 18S-5F (5'-GCGAAAGCATTTGCCAAGAA-3') and 18S-9R (5'-GATCCTTCCGCAGGTTCACCT-3') (Vandergast and Roderick 2003). A portion of the mitochondrial COI gene (658bp) was amplified using the primer pair LC01490 (5'-

GGTCAACAAATCATAAAGATATTGG-3') and HC02198 (5'-TAAACTTCAG

GGTGACCAAAAAATCA-3') (Folmer et al. 1994). For all primers, PCR was performed

in a final volume reaction of 25 µl final volume, adding 9 µl of PCR-grade water, 12 µl of

ReadyMix Taq PCR Mix with MgCl$_2$ (Sigma, St. Louis, MO), 2 µl of DNA template, and

1 µl of each primer. Thermal cycling conditions included: initial denaturation at 94°C for

1 min, 35 cycles of 94°C for 30 sec, 50°C for 40 sec, 72°C for 1 min, and final extension

at 72°C for 10 min. The amplified products were loaded onto a 1.5% agarose gel and

visualized using GelRed^{TM} (Biotium, San Francisco, CA) to confirm amplification. PCR

products were purified using Quantum Prep PCR Kleen Spin Columns (Bio-Rad

Laboratories, Hercules, CA) and sequenced at the Clemson University Genomics Institute

(Clemson, SC). All sequences were checked and edited manually, and contigs were

assembled and aligned in Sequencher 5.2 (Genes Codes Corporation, Ann Arbor, MI). The

18S rDNA sequence was uploaded onto the GenBank database at the National Centre for

Biotechnology Information (http://www.ncbi.nlm.nih.gov) (Table 7.2).

Results

M. cribraria **as Nematode Hosts.** Over 13 sampling dates, 1459 *M. cribraria*

females were collected, of which 402 were dissected (28%), with 13 parasitized by

nematodes (3.2%). The infection rate peak (15%) was reached on 21 July when five

females were sampled for every 20 sweeps. Out of 1402 males collected, 273 were

dissected (19%), with 7 parasitized (2.6%). The peak of infection rate of males (9%) was

reached on 21 July, when males were sampled at four per 20 sweeps. The sex ratio of

sampled adults over the course of the season did not differ from the expected 1:1 ratio (χ^2 = 1.136; df = 1; P = 0.2866). Mermithid infection was not biased towards host sex (χ^2 = 0.529, df = 1; P = 0.4669). The last recorded adult infection was on 9 October, when densities averaged 31.1 adults per 20 sweeps (Fig. 7.1). All juvenile parasitic nematodes were found singly in the abdominal cavity of *M. cribraria* adults.

Out of 808 fifth instars collected, 143 were dissected (18%), with 18 parasitized with a nematode (12.6%). The first infection was recorded on 18 August. A peak of 20% was reached on 27 August, when nymphs were sampled at 25 per 20 sweeps (Fig. 7.1). Infection was recorded in all weeks that nymphs were dissected (n = 5, Fig. 7.1). The last recorded parasitism of a nymph was on 30 September, when fifth instars averaged 0.75 per 20 sweeps. Parasitic juvenile nematodes were always found in isolation in the abdominal cavity (Fig. 7.2).

Pentatomidae Species as Nematode Hosts. Five pentatomid species from four genera were collected and dissected (Table 7.1). Overall, seasonal densities of pentatomids were much lower than those of *M. cribraria* (Table 7.1), with *C. hilaris* as the most abundant species, reaching over three adults and nymphs per 20 sweeps on 30 September. Individual nematodes were observed in an adult *Euschistus* sp. on 27 July (n = 1) and 3 August (n = 1). On 27 August, one nematode was observed in the abdominal cavity of an immature *E. servus*. On 9 October, one nematode was observed in a nymph of *C. hilaris*.

Orthoptera: Acrididae as Nematode Hosts. Nematodes were serendipitously discovered in three immature grasshoppers (Orthoptera: Acrididae) on 21 (n = 2) and 27 July (n = 1), when nymphs were sampled at 0.66 and 0.25 per 20 sweeps, respectively,

resulting in an infection rate of 50 and 33%, respectively. Further orthopteran dissections were not carried out after this date.

Nematode Identification. Examination of parasitic juveniles under the microscope revealed well developed stichosomes, a diagnostic characteristic of the family Mermithidae (Kaya and Stock 1997). Genus and species identification requires adult samples and was, therefore, not possible through morphological examination. Sequencing of portions of 18S rDNA ($n = 6$) and COI mDNA ($n = 5$) was successful. The COI mDNA amplicon from the *E. servus* host was not sequenced successfully and was removed from further analyses. All insect nematodes had identical 18S rDNA and COI portion sequences. A Basic Local Alignment Search Tool (BLAST) through GenBank noted the 18S sequence (703bp) had the closest match to *Agamermis changshaensis* Bao, Luo and Luo 18S rDNA (DQ638908; 99% identity). The COI sequence produced significant alignments with areas from the complete *Hexamermis agrotis* Wang, Bao and Chan and *Agamermis* sp. mitochondrian genome (EF368011 and DQ665656; 79% identity). The absence of a tail appendage on parasitic juveniles and presence of a tail end ring provided robust evidence for an *Agamermis* genus identification (Kaiser 1991).

Discussion

We provide the first report worldwide of a mermithid nematode infecting the immature stages of *M. cribraria*. We also report the first South Carolina mermithid host record for *C. hilaris*, *E. servus,* other *Euschistus* sp., and an orthopteran. Previous pentatomid-mermithid infections in the United States have been reported in nymphs and

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adults of *C. hilaris* [as *Acrosternum hilare* (Say)] in Louisiana (Kamminga et al. 2012) and

E. servus adults in Texas (Esquivel 2011). Literature reporting mermithid infections in

pentatomids and plataspids worldwide is scarce (Table 7.2). Records are often single

observations, and specimens are frequently identified only to the family level due to lack

of obvious morphological features. Furthermore, molecular analysis is rarely carried out;

hence few GenBank reference sequences are available for comparison. Only one

pentatomid-mermithid infection study in the United States assigned genus identification to

the collected mermithid (Kamminga et al. 2012). The presence of a tail appendage on the

parasitic juvenile (Kaiser 1991) confirmed the molecular identification of *Hexamermis*

from GenBank available sequences (Kamminga et al. 2012). Availability of genomic

sequences makes phylogenetic analyses and comparisons possible (Poinar et al. 2007).

Observations that *Allomermis solenopsi* n. sp., a parasite of the fire ant, *Soleopsis invicta*

Buren, requires standing water to emerge from its host was consistent with the placement

of the species as sister taxa to *Mermis* Dujardin, in which some species cause their hosts to

seek open water when they are ready to emerge. Genus identification through molecular

techniques can, therefore, help predict and infer mermithid biology, which can ultimately

assist in rearing protocols, essential if mermithids are to be used for future research and

incorporation into current management protocols.

Through integration of morphological and molecular analyses, we provide evidence

that a species of *Agamermis* infected insect hosts in South Carolina. The same *Agamermis*

species was observed in three insect families across two orders, an observation consistent

with the reported broad host range characteristic for this genus (Poinar 1979, Choo and

Kaya 1990). We observed no mermithid infection in *Podisus maculiventris* (Say), a generalist predator of crop pests (O'Neil 1988). It will be important to investigate whether this pentatomid and other beneficial predators can harbor mermithids, as this could counteract their valuable role in pest population regulation. Although not considered of primary importance in the southeastern United States*, Euschistus* spp. (comprising the 'lesser' brown stink bug complex which excludes *E. servus*) can predominate populations in the Lower Gulf Coast region of Texas, contributing to cotton yield loss (Hopkins et al. 2010, Williams 2015). Crop damage caused by the 'lesser' complex in South Carolina is negligible, but, as a suitable host for *Agamermis*, it allows the nematode to propagate and presumably infect other pest species.

Agamermis* species infecting insects have been reported in North America (Cobb et al. 1923, Christie 1936, Weaver and King 1954), Asia (Kaburaki and Imamura 1932, Choo et al. 1995), Australasia (Baker and Poinar 1995), Africa (Igbinosa 1998), and Europe (Rubtsov 1969). In Korea, *Agamermis unka* Kaburaki and Imamura is a major natural enemy of the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), and to a lesser extent the whitebacked leafhopper, *Sogatella furcifera* (Horvath) (Hemiptera: Delphacidae), and has been widely studied regarding future inoculative releases and conservation approaches to manage pest populations in rice (Choo and Kaya 1994). *Agamermis* species live in the soil and infect hosts from the soil directly or after short migration up a plant stem (Nickle 1981, Choo et al. 1995). Stubbins et al. (2015) hypothesized two possible infection routes for mermithids infecting *M. cribraria* (i) during residency under leaf litter at overwintering or (ii) during residency in soybean fields.

Nematode infection in wingless, less mobile nymphs provides strong evidence that infection occurs directly in the soybean field. Early season soybean colonization by adult *M. cribraria* (as observed in this study) can consist of the overwintered or first generation populations, hence, we cannot rule out nematode acquisition during plataspid overwintering. Although studies have reported mermithid infection from insects collected directly from cultivated crops (Bhatnagar et al. 1985, Kamminga et al. 2012) or traps surrounding crops (Esquivel 2011), mermithids have also been found in hemipterans collected from overwintering sites (Tarla et al. 2012, Tarla et al. 2015). It is thought that infection is often higher during this period, as insects are present in one area for long periods of time.

Despite the central pest role of pentatomids and *M. cribraria* in agriculture in the southeastern United States, the impact of mermithid nematodes on populations is under-explored and potentially undervalued. We observed high prevalence of nematode infection in *M. cribraria,* consistent with the previous year (Stubbins et al. 2015), and report pentatomid-mermithid infection for the first time in South Carolina. Sole use of chemicals for pest management is not sustainable; hence research into alternative strategies for incorporation into a chemical-based management system is required. Biological control research endeavors in the past, for pentatomid and plataspid pests, have focused on exploitation of macroscopically perceptible organisms (Orr et al. 1986, Leskey et al. 2012, Seiter et al. 2014). This study underlines the importance of considering more covert natural enemies as population regulators. Results provide a foundation for future studies into prevalence within agricultural systems. Baseline data from a larger sample of fields and

further understanding of mermithid biology, ecology and host-parasite interacting behavior will be essential to understand how valuable entomoparasitic mermithids are at regulating pest natural populations in southeastern farmscapes.

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Table 7.1. Collection, dissection and infection information for pentatomid species collected in Blackville, SC in 2015 by sweep-net sampling.

Pentatomid species	Life Stage	Total number collected	Total number dissected (% dissected)	Number of weeks collected	Number infected (% infected)
Podisus maculiventris	Adult	8	4 (50%)	4	0 (0%)
	Nymph	0	-	-	-
Euschistus servus	Adult	41	37 (90%)	5	0 (0%)
	Nymph	48	46 (86%)	5	1 (2%)
Other *Euschistus* spp.	Adult	5	5 (100%)	4	2 (40%)
	Nymph	0	-	-	-
Chinavia hilaris	Adult	32	32 (100%)	5	0 (0%)
	Nymph	88	84 (95%)	5	1 (1%)
Nezara viridula	Adult	2	2 (100%)	2	0 (0%)
	Nymph	16	7 (44%)	1	0 (0%)

Table 7.2. Worldwide mermithid host records for Pentatomidae (Hemiptera: Heteroptera) and Plataspidae (Hemiptera: Heteroptera).

Host species family	Host species	Host life stage	Genus	Molecular genus identification	Locality	Reference	GenBank accession number
Pentatomidae	*Aelia acuminata*	?[a]	Undetermined	-	Uzbekistan	Sultanov et al.1990	-
	Aelia rostrata	A[b]	Hexamermis	✕	Turkey	Tarla et al. 2012	-
			Mermis	✕	Turkey	Dikyar 1981	-
	Chinavia hilaris	A/N[c]	*Hexamermis*	✓	US	Kamminga et al. 2012	-
		N[d]	*Agamermis*	✓	US	Present study	KX1773336
	Euschistus servus	A	Undetermined	-	US	Esquivel 2011	-
		N	*Agamermis*	✓	US	Present study	-
	Other *Euschistus* spp.	A	*Agamermis*	✓	US	Present study	KX1773336
	Halys dentatus	?	*Hexamermis*	✕	India	Dhiman and Yadav 2004	-
	Nezara viridula	A	Undetermined	✕	US	Fuxa et al. 2000	-
		N /?	*Pentatomermis*	✕	India	Rubtsov 1977, Bhatnagar et al. 1985	-

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	Platynopus sp.	N	*Hexamermis*	×	India	Gokulpure 1970	-
	Piezdorus guildinii	A	Undetermined	-	US	Kamminga et al. 2012	-
		A/N	*Hexamermis* or *Mermis*	×	Uruguay	Riberiro and Castiglioni 2008	-
	Rhaphigaster nebulosi	?	*Hexamermis*	×	Italy	Manachini and Landi 2003	-
Plataspidae	*Coptosoma mucronatum*	?	*Pentatomermis*	×	Slovakia	Rubtsov 1977	-
	Megacopta cribraria	A/N	*Agamermis*	✓	US	Present study	KX1773336

[a] host life stage not recorded
[b] mermithid found in adult
[c] mermithid found in adult and nymph
[d] mermithid found in nymph
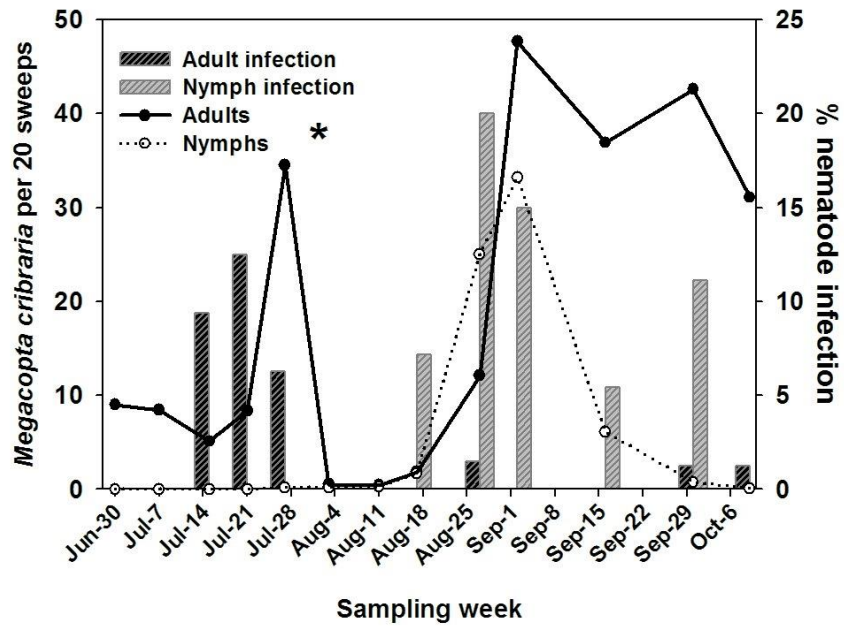
Fig. 7.1. Mean densities of *Megacopta cribraria* adults (male and female data combined) and nymphs per 20 sweeps and percentage of dissected (up to 40 when available) adults and nymphs infected with *Agamermis* sp. Asterisk indicates insecticide application.
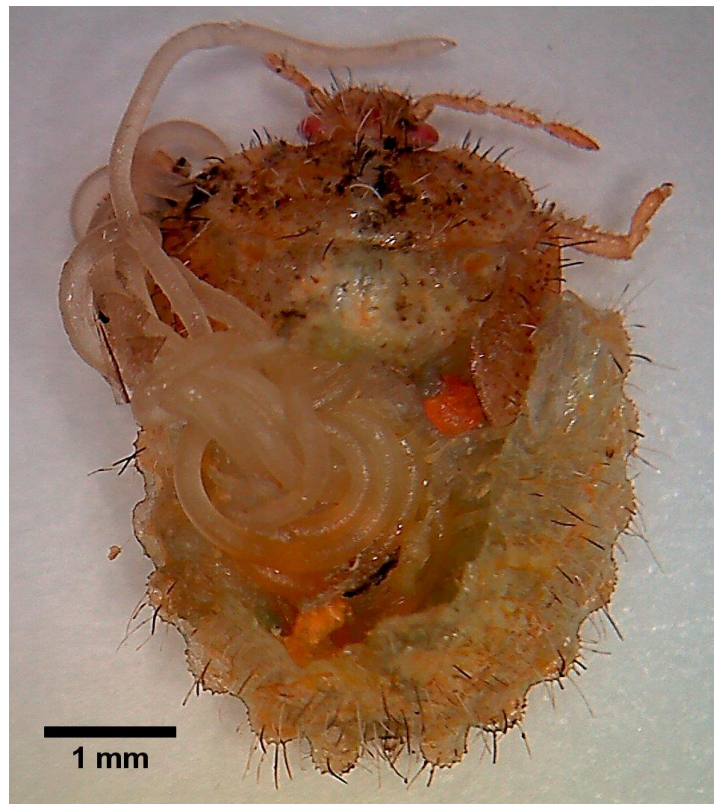
Fig 7.2. Parasitic juvenile nematode within abdomen of fifth instar *Megacopta cribraria* nymph collected from a soybean field near Blackville, SC, during 2015.

CHAPTER VIII

CONCLUSIONS AND FUTURE RESEARCH NEEDS

Invasive insects continue to threaten soybean production in the United States. Within the last fifteen years *Halyomorpha halys* Stål (Hemiptera: Pentatomidae)*, Aphis glycines* (Matsumura) (Hemiptera: Aphididae), *Piezodorus guildinii* (Westwood) (Hemiptera: Pentatomidae), and *Megacopta cribraria* (F.) (Hemiptera: Plataspidae) have added to the complex of insects currently known to damage the crop. This has led to changes in soybean management in many of the production areas affected. The "soybean pest-kudzu beneficial" dichotomy associated with *M. cribraria* did not hinder intense, applied research directed towards optimizing soybean management strategies. Research partnerships between universities and the private sector across geographic regions provided valuable information for researchers, county Extension agents, consultants, farm managers, and homeowners, in a relatively short amount of time. A recent accidental introduction of a third plataspid pest into the Western Hemisphere (specifically Panama) may also benefit from this research in the future.

My research determined the most cost effective *M. cribraria* sampling method for determining population estimates for research or pest management purposes. Sequential sampling plans were generated for beat-cloth and sweep-net sampling, reducing sample sizes required to reach a management decision. Integration of this research into current recommendations in state Pest Management Handbooks would ensure these sampling plans were utilized effectively by soybean growers and Extension agents. Since 2013, there has

been a decrease in *M. cribraria* populations, assigned to various abiotic and biotic factors. Revisiting current threshold recommendations (one nymph per sweep), especially for fields which require multiple applications at this threshold may be useful in the future. Border spray application trials to evaluate the effectivity of reducing *M. cribraria* soybean yield loss as compared with whole field application, is still an avenue to explore and could provide a cheaper option for soybean growers. There is evidence that time of day, soybean stage, personnel executing sampling, weather conditions, and insect behavior can affect sweep-net sampling insect counts. Studies to understand the movement and behaviors of *M. cribraria* within-plant in soybean fields, combined with the other variables associated with sweep-net sampling inconsistencies may aid management decisions in the future.

My research was the first to provide information about the use of cross-vane traps in soybean fields. Although effective at capturing *M. cribraria* adults, traps were limited to qualitative detection of populations rather than as a tool for consistent, indirect measures of field populations. Cross-vane traps may serve as an early-season tool to monitor populations but more studies are needed before robust management recommendations can be defined for specific use. Integration of flight behavior, pheromone, trap design, and lure composition research (some of which is already ongoing) may lead to more effective, reliable detection tools.

I presented baseline information for future work on *M. cribraria* feeding behavior; the activity responsible for the economic importance of this pest. This was the first time a plataspid has ever been recorded using electropenetrography (EPG). Feeding duration and frequency are amongst the many factors that determine crop damage and thus potential

yield loss. It will be important for future *M. cribraria* EPG studies to take into account

differences in feeding behavior between life stages and nymphal instars. Although *M.*

cribraria adults and nymphs are most often found on kudzu and soybean in the US, there

is still uncertainty and contradiction as to which other legume species serve as

developmental hosts, food sources, or merely alighting structures. Furthermore, the role of

non-leguminous plants remains ambiguous. EPG can be a screening tool for possible plant

hosts, and is especially applicable for insects that are recently established or those that are

predicted to invade an area in the future.

Although I provided data regarding the reproductive state of females dispersing into

soybean, there remains a knowledge gap with respect to dispersal capacity and flight

behavior. It will be paramount to investigate these elements to gain a greater understanding

of temporal and spatial dispersal in relation to movement between plants. These studies

would interconnect with identification of all host plants, including bridge-hosts, and the

importance of each host in the bivoltine life cycle of *M. cribraria* that is currently observed

in the Southeast.

Mermithid nematode observations in pentatomid pests and *M. cribraria* is an

example of an understudied but potentially valuable organism that may have the ability to

regulate pest populations. The covert nature of mermithid life history throughout

development, including during the parasitic stage, should not impede further research into

the impact this group of nematodes have on agricultural pests. The importation of such

nematodes may be required in the future to manage invasive species. Although natural

enemies cannot be regarded as a panacea for pest management, they have been known to

contribute to pest population regulation and in some cases reduce pest populations dramatically. The consistent discovery of high *Beauveria bassiana* prevalence in South Carolina serves as another, but more visible example of natural enemies attacking *M. cribraria.* An easier organism to study, propagate, and apply to fields, this entomopathogenic fungus show much promise for the future sustainable management of this invasive soybean pest.

APPENDICES

Appendix A

List of publications

The following chapters have been previously published in refereed journals. These works have been reproduced exactly as they appear in print, with the exception of formatting adjustments to conform to the dissertation guidelines of Clemson University.

Chapter II

Stubbins, F. L., N. J. Seiter, J. K. Greene, and F.P.F. Reay-Jones. 2014. Developing sampling plans for the invasive *Megacopta cribraria* (Hemiptera: Plataspidae) in soybean. J. Econ. Entomol. 107: 2213-2221.

Chapter VI

Stubbins, F. L., P. Agudelo, F.P.F. Reay-Jones, and J. K. Greene. 2015 First report of a mermithid nematode infecting the invasive *Megacopta cribraria* (Hemiptera: Plataspidae) in the United States. J. Invert. Path. 127: 35-37.

Appendix B

Letter of permission to include copyrighted works: Chapter II

Dear Francesca:

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Lisa Junker

Lisa Junker, CAE / Director of Publications & Communications
Entomological Society of America / 3 Park Place, Suite 307 / Annapolis, MD 21401-3722 / USA
Phone: +1 301-731-4535 ext. 3020 / Direct Dial: +1 240-696-3749 / Email: ljunker@entsoc.org

Appendix C

Letter of permission to include copyrighted works: Chapter VI

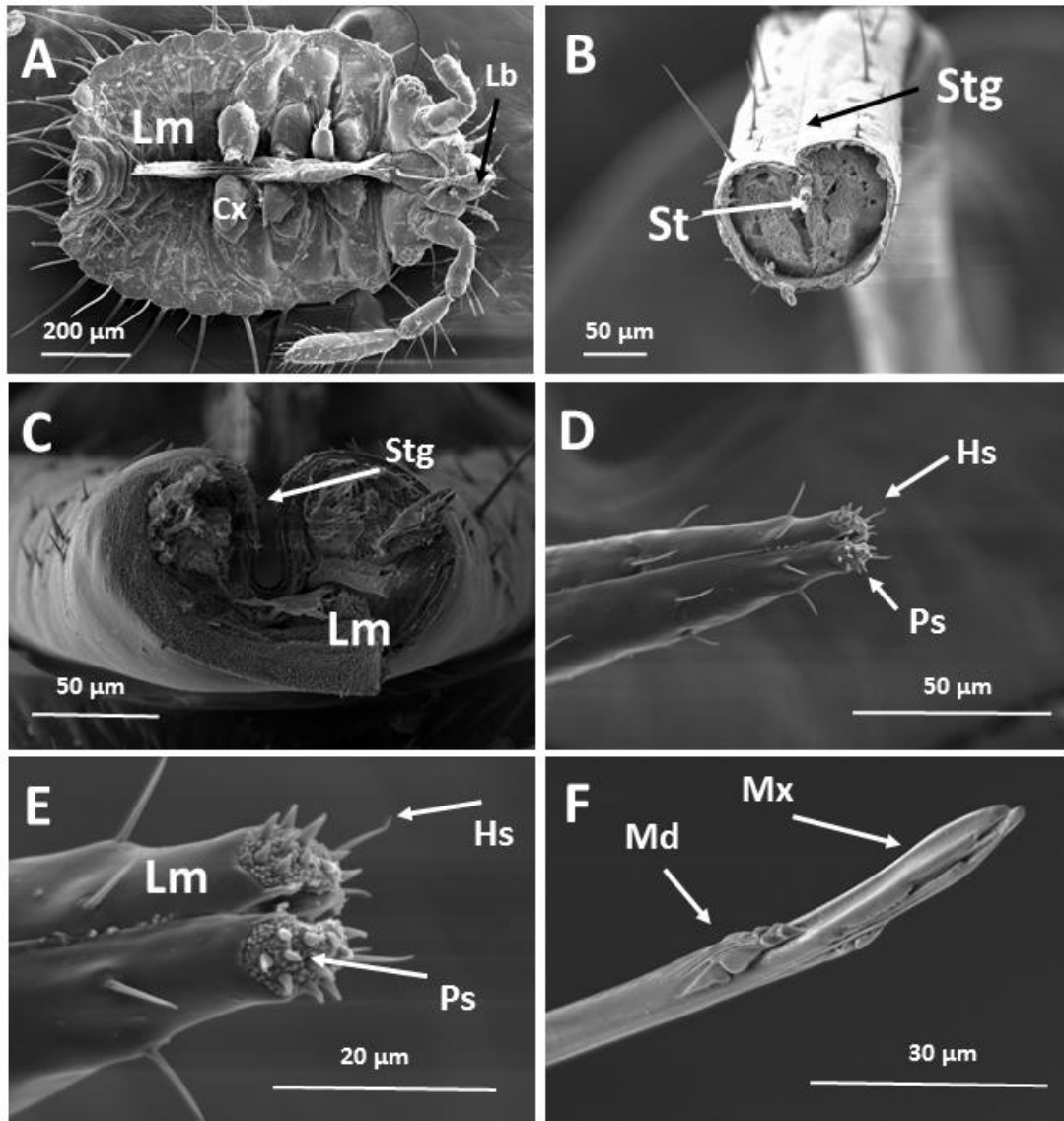Ultrastructure study of *Megacopta cribraria* mouthparts

Fig. D.1. Ultrastructure study of *Megacopta cribraria* first instar nymph mouthparts. Scanning electron microscopy images of (A) Ventral view of whole nymph. Labrum (Lb); labium (Lm); metathoracic coxa (Cx) (B) Cross section through the third labial segment. Stylet (St); stylet groove (Stg). (C) Cross section through first labial segment. Labium (Lm); stylet groove (Stg). (D) Distal end of the labium. Peg-like sensilla (Ps); hair-like sensilla (Hs). (E) Distal end of the labium. Labium (Lm); peg-like sensilla (Ps); hair-like sensilla (Hs). (F) Distal mandibular (Md) and maxillary (Mx) stylets.
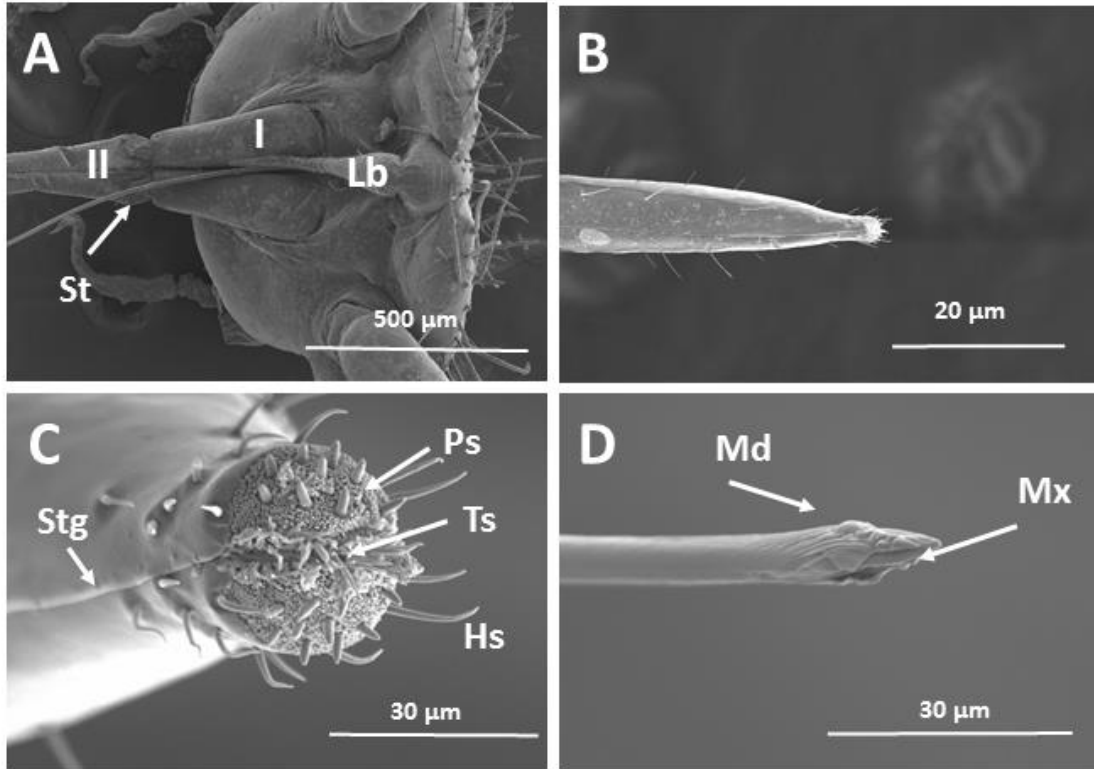
Fig. D.2. Ultrastructure study of *Megacopta cribraria* fifth instar nymph mouthparts. Scanning electron microscopy images of (A) Ventral view of head. Labrum (Lb); first labial segment (I); second labial segment (II); stylet (St). (B) Distal end of the labium (C) Distal end of labium. Stylet groove (Stg); peg-like sensilla (Ps); hair-like sensilla (Hs); tuft-like sensilla (Ts). (D) Distal mandibular (Md) and maxillary (Mx) stylets.
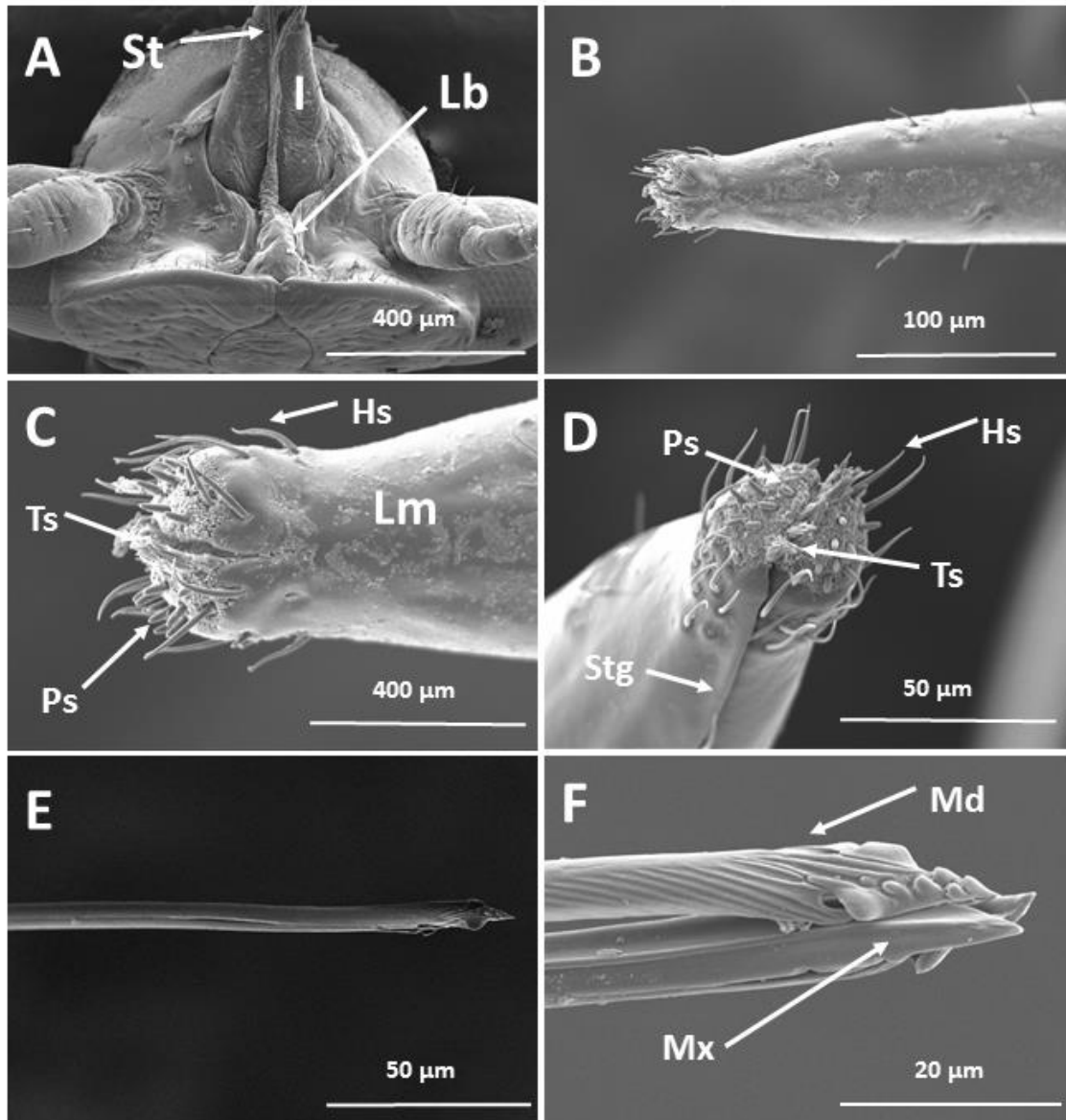
Fig. D.3. Ultrastructure study of *Megacopta cribraria* female adult mouthparts. Scanning electron microscopy images of (A) Head. Labrum (Lb); stylet (St); first labial segment (I). (B) Distal end of the labium (C) Distal end of the labium. Labium (Lm); peg-like sensilla (Ps); hair-like sensilla (Hs); tuft-like sensilla (Ts). (D) Distal end of the labium. Peg-like sensilla (Ps); hair-like sensilla (Hs); tuft-like sensilla (Ts); stylet groove (Stg). (E) Distal end of stylet bundle. (F) Distal mandibular (Md) and maxillary (Mx) stylets.
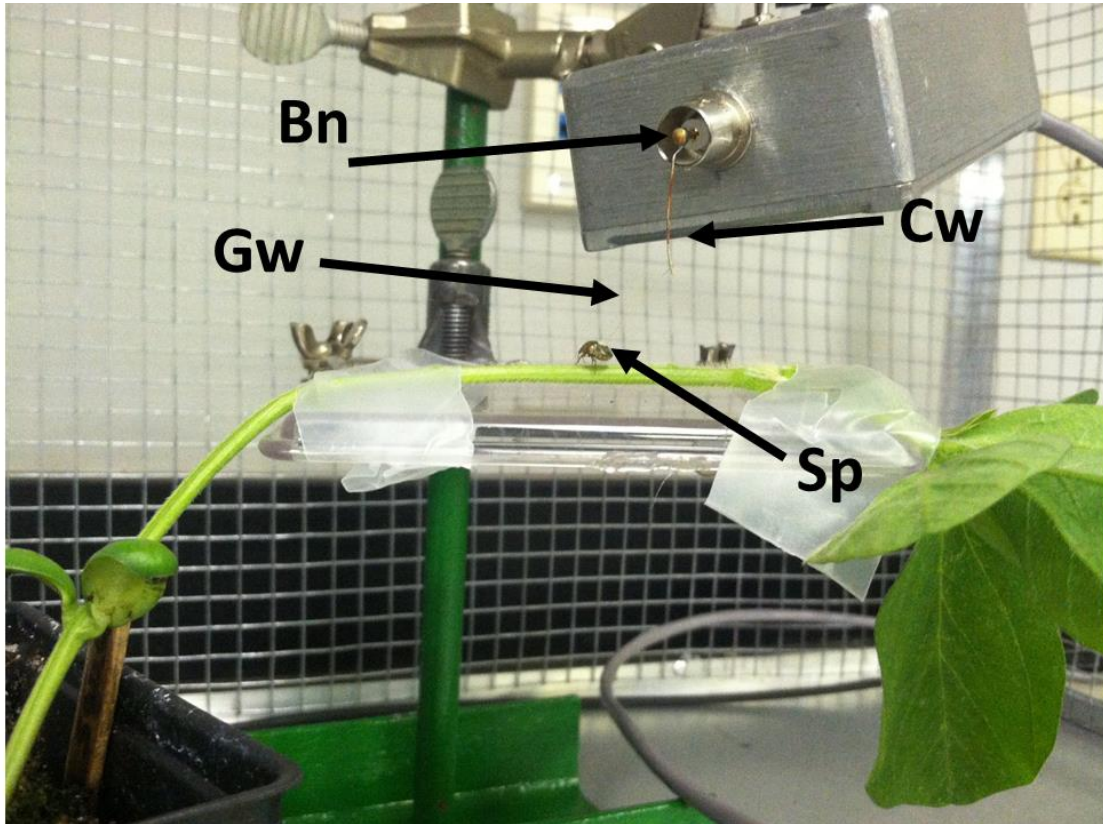
Electropenetrography set up

Fig. E.1. Soybean stem fixed in position with Parafilm strips to a Plexiglass stage with wired female *Megacopta cribraria* placed in a head stage amplifer. Silver paint (Sp); gold wire (Gw); copper wire (Cw); brass nail (Bn).