ABSTRACT

REYNOLDS, DIANE SILCOX. Ecology and Behavior of the Hunting Billbug *Sphenophorus venatus vestitus* in Warm-Season Turfgrass. (Under the direction of Rick L Brandenburg).

The hunting billbug, *Sphenophorus venatus vestitus* Chitenden, is a small, black weevil pest of turfgrass in the United States. Since 2000 there has been an increase in frequency of warm-season turfgrass damage due to hunting billbugs. More information on the biology and behavior of this pest in warm-season turfgrass is necessary to develop an economical and environmentally-friendly management plan. The objectives of this research project were: a) determination of overwintering stage, b) determination of oviposition cycle, c) how soil moisture affects larval development, d) evaluation of synthetic insecticides for control of adults and larvae, e) development of a technique to determine hunting billbug feeding behavior, f) determination of the level of damage produced by adult hunting billbugs in warm and cool-season turfgrass, and g) determination of feeding behavior in warm and cool-season turfgrass using previously mentioned technique.

Overwintering studies were conducted in a bermudagrass stand (*Cynodon* spp.), with a known infestation of hunting billbugs from 2010-2013 in Raleigh, NC. Submersion of turfgrass and soil samples in a salt solution yielded 24 adults, 2 small larvae, 8 medium larvae, and 10 large larvae over all three years. Oviposition studies were conducted weekly for three years, where the number of eggs each female adult hunting billbug oviposited was recorded daily for 7d. Females oviposited consistently for the duration of the collection period in all years. Soil moisture effects on larval development were conducted with sandy

soil at moisture levels of 80, 60, 40, or 20% of total porosity. Medium-sized larvae developed best in containers maintained at levels of 20% of total porosity occupied by water. Greenhouse evaluation of synthetic insecticides against adult hunting billbugs found neonicotinoid and pyrethroid products provide the greatest mortality. Evaluation of synthetic insecticides against larval hunting billbugs found similar results as adult trials, however overall percent control was too low to base recommendations for control.

Adult hunting billbug feeding behavior was determined through the use of digital image analysis and an enzyme-linked immunosorbent assay (ELISA) developed to test for the presence of purified goat IgG treated on either the shoots or roots of the turfgrass plant. Feeding behavior trials using digital image analysis in warm and cool-season turfgrass found that 60% of the warm-season turfgrass trials had differences among billbug treatments, while 12% of the cool-season turfgrass trials had differences among billbug treatments. The data did not indicate a trend in average percent green cover among photograph dates. Feeding behavior trials using an ELISA assay found that the protein was not translocated within the plant or leached into the soil, was able to be removed from the insect exoskeleton, and was detected in the insect gut. Beetles exposed to treated bermudagrass and zoysiagrass shoots had a 50% and 8% chance, respectively, of testing positive for purified goat IgG. Beetles exposed to treated shoots of tall fescue and treated roots of bermudagrass, zoysiagrass (*Zoysia* spp.), or tall fescue (*Festuca arundinacea*) did not test positive.

The results of these studies have provided a better understanding of hunting billbug biology and behavior in warm-season turfgrass. This information has allowed us to develop a management plan that focuses on season-long monitoring of adult hunting billbugs. This will

inform turfgrass a manager when adults become active in the spring, thus indicating that oviposition begins. Tracking rain events after adults have reached peak abundance in the spring will inform managers of larval survival likelihood. If visible damage is associated with billbug populations, a treatment with a pyrethroid or neonicotinoid product when adults have reached peak abundance will maximize effectiveness of application and reduce environmental inputs.

Ecology and Behavior of the Hunting Billbug *Sphenophorus venatus vestitus* in Warm-Season Turfgrass

by
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BIOGRAPHY

Diane Elaine Silcox was born on 11 November 1985 to Dr. Charles Silcox and Linda Hinrichs and older brother Russell Silcox. She grew up in Perkasie, PA and graduated from Pennridge High School in 2004. After graduation she moved to Oxford, OH to attend Miami University. While attending Miami, she rode on the Miami Equestrian team and placed top five in the nation twice at the Intercollegiate Horse Show Association national competition. She also conducted research investigating how ants utilize invertebrate carrion. Upon graduating Miami with a Bachelor of Arts degree in Zoology, she headed south to pursuit her Master's degree under the direction of Dr. Rick Brandenburg. This research focused on the response of the tawny mole crickets (Orthoptera: Gryllotalpidae) to synthetic insecticides and their residues. After completing her Master's in 2011, Diane started her doctorate degree under the direction of Dr. Rick Brandenburg. This research, presented here after, focused on the ecology and behavior of the hunting billbug *Sphenophorus venatus vestitus* in warm-season turfgrass.

On 19 Oct 2013 Diane married Dr. William Casey Reynolds in Cary, NC. Upon completion of her PhD Diane looks forward to joining Casey, her most angelic golden retriever, Ella, and the world's spottiest beagle, Mandy in College Station, Texas. She will seek a career that challenges and motivates her.

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LITERATURE REVIEW

Genus *Sphenophorus*. The genus Sphenophorus contains 71 species of billbugs (Coleoptera: Curculionidae), 64 of which occur throughout the United States, southern Canada, and Mexico. This genus of weevils is generally black or dull red/brown in color, robust and hard bodied, with a long beak-like snout that contains chewing mouthparts at the distal end. Their elbowed antennae consist of a large, bulbous antennal club at the tip and a long scape that inserts at the base of the proximal end of the snout. Characteristics that separate *Sphenophorus* from other related genera include the shape of the antennal club, the relative separation of the coxae, the shape of the mesoepimeron, metaepimeron, and intercoxal processes, the claw segment, and the amount and arrangement of hairs on the underside of the third tarsal segment (Vaurie 1951). Within the genus *Sphenophorus* there are several characteristics to distinguish species of adult billbugs, primarily markings on the pronotum and elytra, color, and relative size (Shetlar 2011). There are currently no published characteristics to distinguish species of larval billbugs. Several characteristics to distinguish species of pupal billbugs are known, primarily setae on various parts, the length of the rostrum, and the width of the pronotum (Satterthwait 1931a).

Within this genus there are eight species of billbugs that are known pests of turfgrass. The bluegrass billbug, *S.parvulus* Gyllenhal, is the most widely distributed of the turfgrass billbugs. Its primary host is Kentucky bluegrass (*Poa pratensis* L.), but it will also feed on perennial ryegrass (*Lolium perenne* L.), tall fescue, fine fescue (*Festuca* spp.), and 14 other grasses not grown for turf. The little billbug, *S.minimus* Hart, is similar in appearance and biology to the bluegrass billbug. Its primary hosts are cool-season turfgrasses. The hunting

billbug, *S. venatus vestitus* Chittenden, is the second most widely distributed of the turfgrass billbugs. Its primary hosts are zoysiagrass (*Zoysia* spp.) and bermudagrass (*Cynodon dactylon* [L.] Pers.), but it will also feed on St. Augustinegrass (*Stenotaphrun secundatum* Kuntze), centipedegrass (*Eremochloa ophiuroides* [Munro] Hack), bahiagrass (*Paspalum notatum*), Kentucky bluegrass, tall fescue, and perennial ryegrass. The Phoenician billbug, *S. phoeniciensis* Chittenden, is limited to southern California and Arizona, where it is the most commonly found billbug species. Its primary hosts are warm-season grasses. The Denver billbug, *S. cicatristriatus* Fabraeus, is found in the Rocky Mountains and northern Great Plains. Its primary hosts are Kentucky bluegrass and perennial ryegrass, but it will feed on all cool-season turfgrasses. The uneven billbug, *S. inaequalis* (Say), is limited to the eastern United States. Its primary host is bermudagrass, but it will also feed on Kentucky bluegrass, perennial ryegrass, and fescues. The relative importance of *S. apicalis* (LeConte) and *S. coesifrons* Gyllenhal in turfgrass is unknown. *Sphenophorus coesifrons* is found throughout the southern United States, where it's primary host is bahiagrass. *Sphenophorus apicalis* has been found in the Gulf States and New Jersey. Its primary host has not been identified, but is has been reported to occur in relatively high numbers on Florida lawns (Johnson-Cicalese 1988, Vittum et al. 1999).

Hunting Billbug *Sphenophorus venatus vestitus* Life History. *Sphenophorus venatus* was first described by Say in 1831 (Vaurie 1951) and was described later as the Y-marked billbug (Satterthwait 1919), which damaged forage and grain crops. Their initial range extended from Maine to Florida and Wisconsin to Texas (Satterthwait 1919). Their initial host-plant list included timothy (*Phleum pretense* L.), bermudagrass, wheat (*Triticum*

spp. L.), American Great Bulrush (*Scirpus validus* Vahl.), and yellow nutsedge (*Cyperus esculentus* L.), which was considered to be its preferred host (Satterthwait 1931a). Upon further examination of *S. venatus*, there were found to be several related species within *S. venatus* and thus *S. venatus* was separated into five subspecies: *S.v. venatus*, *S.v. vestita*, *S.v. glyceriae*, *S.v. confluens*, and *S.v. reticulaticollis* (Vaurie 1951). Three of these, *S.v. venatus* (hunting billbug), *S.v. vestita*, and *S.v. confluens* are considered pests of turfgrass (Vittum et al 1999), with *S.v. vestita* considered the most damaging and abundant.

The hunting billbug was first described as a pest of turfgrass in Florida, when a carton of zoysiagrass sod was found to contain larvae, pupae, and adult hunting billbugs (Kelsheimer 1956). It was apparently introduced into Hawaii in 1960, where it is now found throughout the state, and in southern California, where it was repeatedly intercepted in shipments of zoysiagrass and centipedegrass shipped from Georgia and other eastern states (Tashiro1987). Reports of hunting billbug as a pest in turfgrass were recorded in Florida (Kelsheimer 1956, Huang and Buss 2009), Kansas (Brussell and Clark 1968, Oliver 1984), Nebraska (Baxendale 1990), New Jersey (Johnson-Cicalese et al. 1990), Arkansas (Young 2002), Missouri (D.E. Silcox. personal observation 2010) and North Carolina (Doskocil 2010).

Primary host plants for the hunting billbug include zoysiagrass, bermudagrass, centipedegrass, and St. Augustinegrass. Additional hosts include tall fescue (*Festuca arundinacea* Schreb.), Kentucky bluegrass, perennial ryegrass, Chewings fescue (*Festuca rubra* L. spp. Commutate Gaud), nutsedge (*Cyperus* spp.), crabgrass (*Digitaria* spp. L.), signal grass (*Brachiaria decumbens* Stapf), barnyard grass (*Echinochloa crusqalli* Beauv.),

wheat, corn (*Zea mays* L.), sugarcane (*Saccharum officinarum* L.), Pensacola bahiagrass (*Paspalum notatum* Flugge), and leatherleaf fern (Vittum et al. 1999).

Hunting Billbug Life Cycle. Hunting billbugs overwinter as larvae and adults (Vittum et al. 1999, Shetlar 2003) in sheltered locations around infested turfgrass (Tashiro 1987). In the spring, adults become more active, and the overwintering larvae pupate and then turn into adults. Female hunting billbugs chew holes into stolons of the turfgrass and insert their oblong, pearly white eggs in these holes (Kelsheimer 1956, Shetlar 2003). The eggs hatch 3-10 days after oviposition (Kelsheimer 1956) and the white, legless larvae, with a brown head capsule measuring <1.0mm in width (Doskocil and Brandenburg 2012), begin feeding within the stolon until they become too large for the stem. The larvae will then drop down into the soil and feed externally on the turfgrass plant (Shetlar 2003). The larvae presumably go through 4-5 instars (Huang 2008) and can be found from the thatch layer to 23cm in the soil profile (Doskocil and Brandenburg 2012, D.E. Silcox. personal observation). After 3 to 5 weeks of feeding the larvae form 8-13 mm long, light tan pupae in the thatch layer and down to 15 cm in the soil profile (Kelsheimer 1956, Oliver 1984, Doskocil and Brandenburg 2012). The mahogany to black colored adults emerge in 3 to 7 days and begin to feed and mate (Kelsheimer 1956). The adults measure 8-11mm in length, with a coarsely punctated pronotum, and a smooth, nonpunctated Y-shaped median area with parenthesis-like curved markings on the sides (Satterthwait 1919, Vaurie 1951, Vittum et al. 1999). Hunting billbugs have from one generation per year in northern regions (Vittum et al. 1999), to two generations in transitions zones (Young 2002), and up to six generations in southern regions (Huang and Buss 2008). Adult hunting billbugs are most numerous during the spring

and fall; they are still active, but less numerous in the summer and winter (Vittum 1999). They are most active on the turfgrass surface at night, but occasionally are active during the day. They rarely fly, and instead rely on walking to find suitable host locations and mates (Shetlar 2003). The adults will feign death for short periods of time when disturbed (Vaurie 1951, Oliver 1984). Larvae are most active during the summer and early spring (Vittum 1999, D.E. Silcox. personal observation). In southern regions, hunting billbugs can be found in all stages throughout the year.

Hunting Billbug Damage. Damage caused by hunting billbugs is often misdiagnosed as disease, other insect pests, nematodes, drought stress, fertility issues, delayed spring green-up, or other agronomic factors (Potter 1998, Vittum 1999, Shetlar 2003). Initial indications of billbug damage are yellowing or browning of areas with an appearance similar to fertilizer burn; however, in the case of this insect damage, the turfgrass can be pulled out by the handfuls (Kelsheimer 1956). Damage is frequently seen in early spring, summer (Vittum 1999) and fall (Doskocil 2010). On golf courses, damage is most common in mowed roughs, tee banks, bunker slopes, and other high-sloped, sunny areas (Shetlar 2003). If unmanaged, the damage will progress into larger patches of brown, desiccated turfgrass (Shetlar et al. 2012). Often damage goes undetected in the summer when the turfgrass is actively growing. Adults and young larvae feed on stolons, crowns, and new leaf buds, while older larvae feed on the roots and runners to a depth of 8cm (Vittum 1999). Hunting billbugs most commonly feed on bermudagrass (Satterthwait 1931a, Johnson-Cicalese and Funk 1990, Huang and Buss 2009), zoysiagrass (Kelsheimer 1956, Brussell and Clark 1968, Huang and Buss 2009), and centipedegrass (Tashiro 1987). They also can feed on

orchardgrass (Kamm 1969), St. Augustinegrass (Tashiro 1987), bahiagrass (Vittum 1999), Kentucky bluegrass, tall fescue, perennial ryegrass, and chewings fescue (Johnson-Cicalese and Funk 1990). Diagnosing billbug damage requires using a shovel to cut three sides of a large flap of turfgrass and peeling it back to reveal any larvae present (Shetlar 2003). Since larger larvae can be present from the root zone to 8cm into the soil profile, extra digging may be required (Young 2002).

Hunting Billbug Management. Hunting billbug management is difficult because larvae and adults remain hidden in leaf sheaths, among stems, and in the soil profile. Damage can go unnoticed until their population levels exceed management thresholds. Monitoring adult activity in the spring can help in timing control options. The current treatment threshold when targeting the adult stage is 15 to 25 adult billbugs collected during the day on paved surfaces in a five-minute period (Tashiro and Personius 1970). Since adult activity is occasional during the day other monitoring techniques should be used. The easiest and least destructive is to go out at night with a headlamp and search for adults on the turf surface. A second option is installing pitfall traps by removing a core of turfgrass using a cup-cutter and replacing the core with a 16oz (473 ml) deli cup. Counting adults caught in the trap several times a week will give a good indication as to when control for adults may be warranted (Potter 1998).

Cultural Control. Traditional cultural control practices, such as adjustments in mowing height, thatch management, irrigation, fertilization regime, and optimal soil moisture have not been developed for the hunting billbug. There is evidence suggesting that increasing the mowing height and lowering nitrogen levels significantly increases Kentucky bluegrass

billbug larval densities and turfgrass injury ratings (Bishop et al. 1981). Billbugs prefer less dense turf, such as that found at higher mowing heights, and lower nitrogen rates (Johnson-Cicalese 1988). Presumably the higher nitrogen levels increase the capacity of the turfgrass to recover and thus mitigate billbug damage.

Moisture levels can influence billbug survival and damage propensity (Johnson-Cicalese 1988). Dry conditions during oviposition result in egg desiccation and mortality. Moist conditions during larval development can allow the turfgrass to recover. When coupled with high humidity, high soil moisture can increase the presence of *Beauveria* fungus. An increase in the presence of *Beauveria* could increase control of turfgrass pests by this fungus. Turfgrass plots receiving frequent light irrigation had less billbug damage than plots receiving infrequent heavier irrigation (Johnson-Cicalese 1988).

The use of resistant cultivars is a promising management tactic for minimizing the damaginf effects of hunting billbugs. Several studies have indicated that cultivars of both zoysiagrass and bermudagrass have varying levels of susceptibility to the hunting billbug. In zoysiagrass, damage caused by hunting billbugs is typically greater in Z. japonica cultivars than in Z. matrella cultivars (Reinert and Engelke 2001, Huang 2008, Reinert et al. 2011). Cultivars of zoysiagrass that are the most resistant to hunting billbug include ‘Diamond’, ‘Zorro’, ‘Cavalier’, ‘Royal’(Reinert and Engelke 2001, Reinert et al. 2001), and ‘Cashmere’ (Huang 2008). Cultivars of zoysiagrass that are the most susceptible to hunting billbug include ‘Meyer’ (Reinert and Engelke 2001, Reinert et al. 2011), ‘El Toro’, ‘Palisades’ (Huang 2008, Reinert et al. 2011), ‘Belair’, and ‘Zenith’ (Huang 2008). In bermudagrass, damage caused by hunting billbugs is typically greater in cultivars that have thicker stem

diameters than in cultivars with thinner stem diameters. Cultivars of bermudagrass that are the most resistant to hunting billbug include ‘Tifeagle’ and ‘Tifdwarf’ (Huang 2008). Cultivars of bermudagrass that are the most susceptible to hunting billbug include ‘Tifway’ and ‘Celebration’ (Huang 2008). Turfgrass managers may be able to reduce their pesticide use by selecting less susceptible cultivars when installing or repairing turfgrass areas.

Biological Control. There are numerous parasitoids, nematodes, and fungal pathogens documented to control or minimize damage from hunting billbug populations. Several parasitoids have been identified that utilize hunting billbugs. The first incidence of billbug biological control was a hymenopteran, *Zavipio belfragei* Cresson [renamed *Vipio belfragei* (Cresson)], which was reared from billbug larvae (Satterthwait 1919). The most widely reported parasitoid is a mymarid wasp, Anaphes (Anaphoidea) calendrae Gahan, which parasitizes the eggs of several billbug species (Satterthwait 1931b). This wasp has been introduced and established in Hawaii to reduce billbug populations (Davis and Krauss 1964, Davis and Chong 1968, Beardsley 2000), where it has effectively reduced hunting billbug populations in range grasses, however it is still an occasional pest in turfgrasses. The American toad and numerous bird species have also been reported to prey on billbugs (Satterthwait 1919).

Entomopathogenic nematodes are tiny worms that attack insects by entering through natural openings in the host cuticle. Upon entering a host, nematodes in the genera *Heterorhabditis* and *Steinernema* release bacteria that produce toxins, which kill the host rapidly. The nematodes feed on the bacteria and host tissue, and produce several thousand new nematodes which escape from the dead host in as little as 10 days (Vittum et al. 1999).

Nematodes can provide adequate control of numerous turfgrass pests. There are four species of nematodes, *S. carpocapsae*, *S. feltae*, *H. bacteriophora*, and *H. heliothidis* that have provided mixed results against billbugs (Johnson-Cicalese 1988, Smith 1994, Niemczyk and Shetlar 2000). Treatments of nematodes are most effective when the host larvae are in the crowns of the plants. However, this can be difficult to determine since the larvae would be concealed by the plant and thus undetectable.

Endophytes are fungi associated with certain plant species that grow within the plant and are expressed in highest concentrations in above-ground parts. They produce alkaloids which act as direct toxins and which deter feeding by susceptible insect species. Feeding on entophyte-infected plants causes reduction in insect growth or mortality (Vittum et al. 1999). Endophytic fungi in the genera *Acremonium* and *Neotyphodium* are known to be effective against hunting billbugs. Survival of adult billbug species was reduced when they fed on tall fescue with endophytes; however there was little difference in the amount of feeding on tall fescue with and without endophytes (Johnson-Cicalese and White 1990). Larval survival also decreases in tall fescue with endophytes (Murphy et al. 1993). Survival and feeding activity of adult hunting billbugs was also reduced when they fed on perennial ryegrass with endophytes (Johnson-Cicalese et al. 1989, Huang 2008).

Every major group of turfgrass insect pests is susceptible to various fungal pathogens. Fungal spores adhere to the body of an insect, and, when conditions are favorable, the spores will germinate, and the hyphae that emerge from the spore penetrates the insects' cuticle to invade the circulatory system. The fungus produces a toxin that quickly kills the insect. Once the host is dead, hyphae emerge from the insect and develop conidiphores that produce

infected spores. Beauveria spp. is the only naturally occurring pathogen reported in billbug populations (Johnson-Cicalese 1988, D.E. Silcox. personal observation). A biopesticide containing Beauveria has been marketed, but there is little information available on the level of billbug control (Potter 1998).

Chemical Control. Insecticides are the primary tool for management of turfgrass insect pests (Shetlar et al. 2012). The key to effective use of insecticides is timing the application to correlate with the particular insects life cycle that produces the greatest likelihood of control. This can often be difficult with subterranean insects, such as the hunting billbug, which spend all or the majority of their life cycle underground. Therefore, a thorough knowledge of the insect's life cycle is needed in order to maximize insecticide efficacy. Current recommendations for hunting billbug control suggest an insecticide application in the spring to prevent or suppress billbug populations (Shetlar et al. 2012). This is a general guideline and can vary by geographical region. In warmer regions, like the Southeast, multiple generations a year and mild winters (temperatures primarily above freezing) could change the timing of these spring applications. It is best to monitor adult populations throughout the year in order to make an appropriately timed insecticide application. Larval control can be difficult with this insect because it can burrow as deep as 22.86 cm (D.E. Silcox personal observation) when soil moisture is not optimal. Targeting larval populations is often considered an alternative approach to reducing adult populations (Niemczyk 1983).

The majority of the insecticide trials on adult hunting billbug control have been conducted on Kentucky bluegrass stands in a field setting. These studies applied various

insecticides to the turfgrass surface, applied irrigation after treatment, and sampled for billbugs by removing cores from the center of each plot and counting the number of billbug larvae and pupae present. The results from these studies are summarized as follows:

Table 1. List of active ingredients, rate, percent hunting billbug control and citation

Active Ingredient	Rate	Percent Hunting Billbug Control	Citation
Bifenthrin	116.5 g ai/A	82.7	Heller et al. 2007a
Chlorantraniliprole	116.5 g ai/A	76.0	Heller et al. 2007a, Heller et al. 2007b, and Heller et al. 2008
Chlothianidin	336.2 g ai/A	94.1	Eickhoff et al. 2006
Imidacloprid	336.2 g ai/A	74.75	Heller et al. 2007a, Heller et al. 2008
Thiamethoxam	502.7 ml/A	100	Pierson et al. 2007

Few insecticide trials on adult hunting billbug control have been conducted in warm-season turfgrass. One study on bermudagrass in a field setting found more billbug larvae in the treated plots than in the untreated check (Walker and Royer 2001). Another study on zoysiagrass in a field setting found that the majority of treatments had more billbug adults than in the untreated check (Doskocil et al. 2012). This same study found that an application of Allectus™ (bifenthrin + imidacloprid) applied in the fall or fall and spring effectively reduces billbug populations. These studies suggest that control efforts that target larval populations are most effective, however adults can be targeted if necessary.

There has been one study to assess the lethal dose (50/95) and lethal concentration (50/95) of various insecticides on adult hunting billbugs (Doskocil et al. 2012). In the lethal dose trials there were no differences in activity of bifenthrin and combination products (bifenthrin + imidacloprid or bifenthrin + chlothianidin). When evaluated alone (not in combination) imidacloprid had greater toxicity than chlothianidin. In the lethal concentration trials there were no differences in efficacy between products. There were numerical differences of greater mortality for the combination products when compared to bifenthrin alone. The concentration of neonicotinoid insecticides necessary to elicit 95% mortality differed from those products containing bifenthrin. These results can help a turfgrass manager select the most effective product for their pest complex.

Soil Moisture Effects on Insect Development. Soil moisture is a poorly understood factor that has great consequence on insect life histories. Fully understanding how soil moisture affects insect development not only has ecological significance, but also has a bearing on monitoring and managing insect pests. Three major hypotheses surround the role of moisture as a seasonal factor over an insect's entire life cycle. Soil moisture can either 1) act as a token (anticipatory) stimulus that induces, maintains, or terminates diapause, 2) act to modulate development or activity (accelerate, decelerate, or limit rates of growth, maturation, oviposition, or behavior), or 3) act as a primary stimulus for crucial seasonal activities in insect life cycle (hatching, molting, mating, or movement) (Tauber et al. 1998). Within each of these hypotheses, moisture can act in a quantitative manner or as an all-or-none stimulus. Conducting experiments that focus on determining the role of soil moisture in insect development is often difficult and can be hampered by three crucial issues. First, isolating the

effects of moisture on specific phases of the life cycle is difficult due to the insect's continuously changing physiological state. Field-based knowledge of an insect's relative seasonal changes is needed to accurately determine the effects of moisture on that species, during that particular season. The second critical issue concerns establishing how insects perceive and respond to moisture in their environment. Insects can either respond to moisture as an absolute measure of a seasonal stimulus or as a change in level of stimulus, and these responses can be all-or-none or graded. Third, the technical issues with designing and implementing experiments on moisture are often too great to overcome or accurately describe the insect response (Tauber et al. 1998).

The majority of turfgrass insect pests spend part or all of their life cycle underground. Thus, soil moisture is a key component in their growth and development. Several experiments using turfgrass insect pests have investigated soil moisture effects on oviposition behavior, survival, and the development of eggs, larvae, and adults.

Oviposition behavior. When given a choice, Japanese beetles, *Popillia japonica*, and rose chafers, Cetonia aurata, prefer to oviposit in soils with 20% moisture over soils ranging from 5-15% moisture (Allsopp et al. 1992). Southern masked chafers, *Cyclocephala lurida*, oviposited more eggs in soil maintained at 25.5% soil moisture than in soils ranging from 5-19% moisture; they did not oviposit in air-dried soils (Potter 1983). Green June beetles, *Cotinis nitida*, laid more eggs in soil with moisture levels just below field capacity (20% for the soil tested) than soils above or below field capacity; they did not oviposit in dry soils (Gaylor and Frankie 1979). Mole crickets oviposited more eggs in 12% soil moisture than in soils with 2 or 7% soil moisture. A greater percentage of females oviposited in 10% soil

moisture than in soils with 4 or 7% soil moisture (Hertl et al. 2001). In general, turfgrass insect pests prefer to oviposit in soils with 20% moisture.

Egg survival and development. Japanese beetle eggs did not survive in completely dry sandy or loam soils. In clay soils, moisture above 6% was required for survival (Régnière et al. 1981). Once embryonic development is well underway in Japanese beetle eggs, they become more resistant to moisture extremes (Régnière et al. 1981). Southern masked chafer eggs developed normally at soil moistures at or above wilting point (12.5, 19, and 25.5%), but die in drier soils (Potter 1983). June beetle (Phyllophaga crinite) eggs hatched statistically equal in soils ranging from 0-25% moisture, however no eggs hatched in the 47% soil moisture (Gaylor and Frankie 1979). In general, turfgrass insect eggs survive best in soils with medium levels (12-25%) of moisture; there is a greater mortality of eggs in dry and wet soils.

Larvae survival and development. Japanese beetle grubs that hatched on 3% soil moisture were smaller than other grubs. Under field conditions, these grubs could be more subject to accidental death when they are trying to reach roots to begin feeding (Régnière et al. 1981). June beetle grubs had a greater survival rate in soils that contained 10-30% moisture than soils with high or no soil moisture (Gaylor and Frankie 1979). In general, turfgrass insect larvae survive best in soils with medium levels (12-25%) of moisture; there is greater mortality of larvae in dry (<12%) and wet (>25%) soils.

Adult survival. Japanese beetle adults do not survive in soils without moisture, but they do survive in soils ranging from 5-20% moisture (Allsopp et al. 1992). Mole cricket adults do not survive in soils with 2% moisture, but they do survive in soils ranging from 4-

12% moisture (Hertl et al. 2001). In general, turfgrass insect adults survive best in soils with medium levels (12-25%) of moisture, there is greater mortality of adults in dry and wet soils.

Soil moisture effects on Curculionidae. One study has been conducted on soil moisture effects in the family Curculionidae (Lapointe and Shapiro 1999). This study recorded the cumulative pupation of 68-, 105- and 180-d-old larva. Total pupation increased with increasing soil moisture and plateaued at 60-65% moisture. Optimal soil moisture for pupation ranged between 50 and 70% with a large increase in mortality at 80%. Mortality was also high at 20% soil moisture. The time required for pupation did not differ between soil moisture treatments.

Insect Gut Content Analysis. Understanding the basic biology of insect pests includes an understanding of their feeding habits. Ecologists have been exploiting molecular gut content assays to study these behaviors (Fournier et al. 2008). There are numerous quantitative and qualitative methods for determining the composition of insect guts. Among the most sensitive are the enzyme-linked immunosorbent assays (ELISAs). Enzyme-linked immunosorbent assay is a versatile and highly sensitive test for qualitative or quantitative analysis of antibodies present in a sample (Paulie et al. 2006). ELISA procedures are relatively simple to follow and can be used for single samples and for high-throughput screening. In general, ELISA procedures involve a specific interaction between an antibody and antigen, with one of them immobilized to a solid support (Paulie et al. 2006). This immobilization makes it possible to measure the binding of specific antibodies. The binding of specific antibodies allows for detection and quantification of antigen (Paulie et al. 2006). These interactions are visualized by using enzyme-conjugated reagents and a chromogenic

substrate that provides a color change proportional to the amount of bound antigen (Paulie et al. 2006). There are three main methods for all ELISAs: indirect ELISA, direct ELSIA, and sandwich ELISA (Crowther 2009). Indirect ELISAs are most suitable for screening of sera or other biological fluids. The antigenic components are from bacteria, viruses, allergens, or target antigens for autoimmune reactions (Paulie et al. 2006). Direct ELISAs are useful for quantitating soluble antigens and studying antigenic specificities of any molecules for which antibodies are available. They are particularly useful for analysis of smaller molecules which cannot be analyzed by the more sensitive sandwich ELISA (Paulie et al. 2006). Sandwich ELISAs are useful for quantifying any soluble molecule to which antibodies can be raised. It is the most sensitive of the ELISAs (Paulie et al. 2006).

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Ecology and Management of the Hunting Billbug (Coleoptera: Curculionidae) in Warm-Season Turfgrass

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Abstract

Billbug biology and ecology in cool-season turfgrass has been well documented. However with the increase of billbug incidence in warm-season turfgrass, specifically the hunting billbug *Sphenophorus venatus vestitus* Chittenden, continued research is necessary for effective pest management. Field and laboratory studies were conducted with the hunting billbug to determine the overwintering stage, oviposition timing, effects of soil moisture on larval development, and insecticide efficacy against larvae and adults. Hunting billbugs overwinter as adults, and early-, mid-, and late- instar larvae. Adults become active in March and begin to oviposit, which continues through October. Larvae develop optimally with 20% of the total pore space occupied by water; while increases in percentage of pores occupied by water cause significant mortality. Pyrethroid and neonicotinoids classes of chemistry were effective against adult hunting billbugs. They were also effective against larval hunting billbugs, however overall mortality was low. Management plans for hunting billbugs in warm-season turfgrass should focus on monitoring adult activity and treating when they reach peak abundance.

Keywords *Sphenophorus venatus vestitus*, hunting billbug, warm-season turfgrass, biology, ecology

Turfgrass systems are associated with numerous insect pests that often require monitoring, identification, and management. To create an integrated pest management plan, turfgrass mangers must have relevant and up-to-date information on all turfgrass insect pests. When there are research gaps on pest biology and ecology, damage often gets misdiagnosed and the pest can become a widespread problem (Potter 1998). The hunting billbug in warm-season turfgrass is a recent example of how a lack of basic biology and ecology information can cause widespread turfgrass damage.

The hunting billbug has traditionally been a problem in cool-season turfgrass as a complex of five billbug species (Johnson-Cicalese 1988). Billbug biology and ecology in cool-season turfgrass has been well documented (Vittum et al. 1999). Adult billbugs overwinter in leaf litter and pine straw on the edge of the turfgrass. When temperatures increase in the spring, the adult billbugs will migrate towards the turfgrass and begin feeding, mating, and ovipositing. Female billbugs notch the stems and blades of the turfgrass for oviposition. The small larvae hatch and feed within the stem. When larvae outgrow the diameter of the turfgrass stem, they drop to the thatch and soil where they feed on crowns, stolons, and roots. Damage appears on the turfgrass surface as yellowing areas that spread into larger patches of brown turfgrass (Shetlar et al. 2012). Since, in cool-season turfgrass, billbugs are found as a complex of five species it is difficult to associate the biology and ecology data from these regions with hunting billbug populations in warm-season turfgrass.

Since 2000, there has been an increased incidence of turfgrass damage caused by the hunting billbug as a stand-alone pest in warm-season turfgrass (R.L.B. personal observation).

Billbug biology and ecology studies in warm-season turfgrass are variable between geographical locations. It is unclear in which stage and where overwintering takes place in warm-season turfgrass. Damage in warm-season turfgrass appears most significant in late spring and fall and is often misdiagnosed as disease, drought, nutrient deficiency, delayed spring green-up, dormancy, or other factors (Potter 1998). In Florida, adult billbugs are active all year long, with peak activity in Mar and Apr (Huang and Buss 2009). In Arkansas, adult billbugs are active from Mar through Nov, with peak activity in Mar and Apr (Young 2002). In North Carolina, adult billbugs are active from Mar through Nov, with peak activity in Apr, May, and Sep (Doskocil and Brandenburg 2012). Female billbugs have mature eggs present in their reproductive tract all year long in Florida (Huang and Buss 2009) and from Apr through Oct in Arkansas (Young 2002). Overwintering behavior, oviposition timing, and the effect of environmental factors on development of larva have yet to be studied.

Management of the hunting billbug in warm-season turfgrass primarily consists of multiple applications of insecticides after visible turfgrass damage is present. There have been few insecticide trials in warm-season turfgrass for control of hunting billbug (Walker and Royer 2001, Buss et al. 2004, Doskocil et al. 2012). These trials investigated application timing in reducing field populations of adult and larval hunting billbugs. However, there have been no studies that have targeted insecticide applications to and evaluated mortality of a specific hunting billbug life stage.

In order to create a management plan for the hunting billbug that focuses on environmental safety and cost effectiveness, gaps in knowledge of their biology and ecology

must be filled. The objectives of this study were to determine in what life stage hunting billbugs overwinter, when the adult female hunting billbugs oviposit, how soil moisture effects development of larval hunting billbugs, and the most effective insecticides for management in warm-season turfgrass.

Materials and Methods

Overwintering. Field experiments were conducted at the North Carolina State University Club Golf Course in Raleigh, NC. The area selected consisted of bermudagrass *Cynodon dactylon x C. tranvaalensis* (L.) Pers that had a natural hunting billbug infestation. Samples were collected on 29 Mar 2011, 23 Feb 2012, and 1 Mar 2013 with soil temperatures at 5 cm of 5.5°C, 11°C, and 6.6°C respectively. A 0.91 x 0.91 m area (0.828 m^2 total area) was measured and within this block nine, 0.3 x 0.3 m plots were marked. A sod cutter was used to remove the sod, with thatch and roots attached, from each plot. After the sod was removed from each plot the exposed soil surface was inspected for hunting billbugs. The sod from each plot was placed separately in 9-l plastic container and transported back to the laboratory. A 15-cm deep section of soil from beneath the turfgrass in each plot was removed with a shovel and placed separately in a 9-l plastic container and transported back to the laboratory. In the laboratory, a solution of 1,244 g table salt (Morton Salt, Inc. Chicago, IL) dissolved in 3.78 l of water (~32% salt solution) was poured over the sod or soil until the samples were completely submerged. The submerged samples were monitored for one hour and any billbugs that emerged were recorded. After one hour, the salt solution was removed from the container and the turfgrass was destructively sampled by pulling it apart into

individual plants and searching for any remaining billbugs. The remaining soil was placed in a sieve with a screen diameter of 0.64 mm stacked on a second sieve with a screen diameter of 0.32 mm (Science First®, Yulee, FL). Water from a hose was used to rinse the soil through the sieve. Any remaining billbugs were recorded.

Oviposition Timing. Ten adult female billbugs were collected by hand 30 min after sunset from the turfgrass surface in Raleigh, NC. Collections were made weekly from Jun–Sep 2010, Jul-Sep 2011, Mar-Oct 2012, and Mar-Oct 2013. Each female was held individually in a 30 ml plastic cup (Dart Container Corporation, Randleman, NC) with a 1g square of general lepidopteran diet (Bio-Serv®, Frenchtown, NJ) and a 1.27cm piece of cotton dental wicking (Tidi Products, Neenah, WI) soaked in water (Figure 1). A lid was placed on the container to prevent adult escape. Containers were checked daily for 7d and the number of eggs deposited were recorded. Eggs were removed from the containers with a fine tipped paint brush and placed on a piece of moist filter paper in a 30 ml plastic cup (Dart Container Corporation, Randleman, NC). A lid was placed on the container to prevent moisture loss. In 2012 and 2013, percent egg hatch was recorded for each female and after 7d the females were frozen for dissection to count eggs remaining in the reproductive tract.

Data were subjected to ANOVA (Proc GLM) to determine treatment effects with month nested within year (SAS 9.2 program, SAS Institute 2003). Treatments were subjected to Fisher's LSD test at the 0.05 probability level when *F*-tests indicated significant treatment effects.

Soil Moisture. Soil used in this study is classified as a Candor sand (Sandy, siliceous, thermic Arenic Paleudult, pH = 6.2, Humic Matter % = 0.51%, Cation Exchange Capacity = 3.8) collected from the Sandhills Research Station in Jackson Springs, NC (USDA-NRCS Soil Survey Division). The soil was oven-dried in a 30 cm x 30 cm x 7.6 cm metal container for 24 h before being divided and transferred to 30 ml plastic cups (Dart Container Corporation, Randleman, NC). Relative bulk density for the soil in each cup was calculated by dividing the weight of the soil (g) by the volume of the cup (cm^3). After determining bulk density, porosity of each soil was calculated using the following equation: Porosity = 1 – Bulk Density/ Particle Density, where particle density of oven-dried sand is assumed to be 2.65 g/ml (Brady and Weil, 1996). The total amount of pore space in each container was calculated by multiplying the porosity of soil in each container by the volume of the container. The volume of water calculated to achieve various soil moisture levels (80, 60, 40, and 20% of porosity) was then applied to its respective container using a pipette and mixed to achieve uniform distribution. A medium-sized hunting billbug larva (head capsule width of 1.0-1.7 mm) was weighed and then placed individually in each container. The larvae were placed in a one cm wide by half cm deep depression in the soil and then covered with the sand that was displaced when the hole was made. A lid was placed on each container to maintain the various soil moisture levels throughout the experiment. Containers were checked once every 7d for the larval life status and larval weight. If the billbug was alive after 7 d, it was placed into a new container at the same soil moisture level. Containers were checked every 7d for three weeks. Data (% mortality) were transformed (*binary*) prior to analysis for soil moisture effect using Proc Glimmix (SAS program 9.2, SAS Institute 2003).

Adult Insecticide Trials. Clear plastic deli containers (950 ml, Tripak Industrial USA, LLC, White Plains, NY) were modified with 5 drainage holes (0.5 cm diameter) made in the bottom with a soldering iron. A piece of fiberglass screen (0.027cm mesh dia., New York Wire Company, Mount Wolf, PA) was attached with adhesive to the bottom to prevent insect escape. Containers were filled with a homogenous 1:1 mixture of sterilized all-purpose sand and Fafard® 4P potting mix (Sun Gro® Horticulture, Agawam, MA). A piece of bermudagrass (*C. dactylon x C. tranvaalensis*) sod was cut to fit into the container and allowed to establish in the greenhouse for one month. The turfgrass was irrigated by hand three times weekly to field capacity, mowed once a week at 3.81 cm and fertilized once a month with Miracle-Gro® all-purpose plant food, 20-20-20 analysis (The Scotts Company, Marysville, OH). Adult hunting billbugs were collected by hand at night prior to initiation of insecticide trials from the North Carolina State University Faculty Club Golf Course in Raleigh, NC. Billbugs were held in deli containers (950 ml, Tripak Industrial USA, LLC, White Plains, NY) with a moistened paper towel until introduction into containers the following morning. In 2011, for three separate trials, treatments were applied to the turfgrass surface on 8 Jul, 18 Aug, or 9 Sep and consisted of four replicates of an untreated control, bifenthrin (Talstar® P, FMC Corp., Philadelphia, PA) at 0.229 l ai/ha/ha, chlorantraniliprole (Acelepryn®, Dupont, Wilmington, DE) at 0.0957 l ai/ha/ha, chlothianidin (Arena™ WDG, Valent Biosciences, Libertyville, IL) at 0.0734 g ai/ha, experimental product HGW (Dupont, Wilmington, DE), at 0.0957 l ai/ha/ha, imidacloprid (Imidacloprid 2F, Quali-Pro, Pasadena, TX) at 0.240 l ai/ha/ha, and indoxacarb (Provaunt®, Dupont, Wilmington, DE) at 0.137 l ai/ha/ha was added to the treatment list for the 18 Aug and 9 Sep treatment dates. In 2012,

treatments were applied on 3 May, 11 May, and 26 May and consisted of four replicates of an untreated control, bifenthrin (Talstar® P, FMC Corp., Philadelphia, PA) at 0.229 l ai/ha/ha, chlothianidin (Arena™ WDG, Valent Biosciences, Libertyville, IL) at 0.0734 g ai/ha, imidacloprid (Imidacloprid 2F, Quali-Pro, Pasadena, TX) at 0.240 l ai/ha/ha, bifenthrin + imidacloprid (Allectus ® SC, Bayer CropScience, Kansas City, MO) at 0.470 l ai/ha/ha, bifenthrin + chlothianidin (Aloft™ GCSC) at 0.140 l ai/ha/ha, and dinotefuran (Zylam® Liquid, PBI/Gordon Corporation, Kansas City, MO) at 0.0120 l ai/ha/ha on 3 May and 0.021 l ai/ha/ha on 11 May and 26 May.

For all studies, five billbug adults were placed on the turfgrass surface and allowed to burrow into the soil before treatments were applied. An aqueous preparation of each treatment was applied using a Delta Orbital 360 Sprayer (Delta Industries, King of Prussia, PA) calibrated to deliver 1 ml of solution per handle pull. The containers were irrigated with 10 ml water evenly distributed by hand from a graduated cylinder after treatment and every 2 days for the duration of the study. After treatment, a piece of tulle fabric was secured over the top of the sod with a rubber band to prevent adult escape. Contents of the containers were destructively sampled 7 DAT and alive and dead adults were located and recorded.

Larval Insecticide Trials. Clear plastic containers (950 ml, Tripak Industrial USA, LLC, White Plains, NY), with 5 drainage holes (0.5 cm diameter) and a screen attached with adhesive to the bottom to prevent insect escape, were filled with a standard cup-cutter core of centipedegrass (10.16 cm dia.) from Neuse River Sod Farm in Arapahoe, NC (fine loamy sand). Cores were places in the containers on the same day as treatment, with the exception

of the 3 Apr date, where the cores were removed 2 weeks prior and placed in a greenhouse at North Carolina State University in Raleigh, NC to accelerate spring green-up. Billbug larvae were collected from the same location as the soil cores. Centipedegrass (*Eremochloa ophiuroides*) was cut into strips using a sod cutter and medium to large larvae were collected manually from the bare soil. In 2012, for three separate trials, treatments were applied to the turfgrass surface on 3 Apr, 24 Apr, or 1 May and consisted of four replicates of an untreated control, bifenthrin (Talstar® P, FMC Corp., Philadelphia, PA) at 0.229 l ai/ha/ha, chlorantraniliprole (Acelepryn®, Dupont, Wilmington, DE) at 0.0957 l ai/ha/ha, chlothianidin (Arena™ WDG, Valent Biosciences, Libertyville, IL) at 0.0734 g ai/ha, and imidacloprid (Imidacloprid 2F, Quali-Pro, Pasadena, TX) at 0.240 l ai/ha/ha.

For all studies, five billbug larvae were placed individually in a 1 cm hole created by the blunt end of a pen, and allowed to burrow into the soil before treatments were applied. An aqueous preparation of each treatment was applied using a Delta Orbital 360 Sprayer (Delta Industries, King of Prussia, PA) calibrated to deliver 1 ml of solution per handle pull. The containers were irrigated with 10 ml water after treatment and every 2 days for the duration of the study. After treatment a piece of tulle fabric was secured over the top of the sod with a rubber band to prevent adult escape. Contents of the containers were destructively sampled 7 DAT, remaining larvae were located and percent mortality was recorded.

All insecticide trial data were subjected to ANOVA (Proc GLM) to determine treatment effects (SAS program 9.2, SAS Institute 2003). No treatment x trial interaction occurred for the adult or larval insecticide trials and therefore data for each run within each

year were pooled. Treatments were subjected to Fisher's Protected LSD test at the 0.05 probability level when *F*-tests indicated significant treatment effects.

Results

Overwintering. In 2011, 7 adults and 15 larvae were collected from the bermudagrass area. Of those larvae, 8 were large (head capsule width >1.7mm), 6 were medium (head capsule width 1.0-1.7mm) and 1 was small (head capsule width <1.0mm) (Table 1). In 2012, 8 adults and 5 larvae were collected from the bermudagrass area. Of those larvae, 2 were large, 2 were medium, and 1 was small (Table 1). In 2013, 9 adults and no larvae were collected from the bermudagrass area (Table 1). For all sampling dates, no pupae were found.

Oviposition Timing. There were no differences in number of eggs oviposited by female billbugs among years ($P = 0.9261$) (Figure 2); total oviposition each year ranged from 105-251 eggs. There were no differences in number of eggs oviposited by female billbugs among months within respective year ($P = 0.0769$) (Figure 2); May had the highest total number of eggs oviposited (120 eggs) and Oct had the lowest total number of eggs oviposited (30 eggs). In 2012, there were no differences in percent egg hatch among months ($P = 0.9752$) (Figure 3); average percent hatch ranged from 52.3 – 88. In 2013, there were no differences in percent hatch among month ($P = 0.5446$) (Figure 3); average percent hatch ranged from 44 - 70. In 2012, there were no differences between number of eggs in the reproductive tract by month ($P = 0.5824$) (Figure 4); Mar had the highest total number of eggs in the reproductive tract (119) and Sep had the lowest number of eggs in the

reproductive tract (59). In 2013, there was a difference in the number of eggs in the reproductive tract by month ($P = 0.0001$) (Figure 5); May had the highest total number of eggs in the reproductive tract (200) and Mar had the lowest number of eggs in the reproductive tract (3).

Soil Moisture. There was a difference in larvae hunting billbug mortality among soil moisture treatments ($P < 0.0001$). After 7d, the soil moisture treatment where 80% of the total pores were occupied by water had the highest mortality (63.1% mortality), followed by 60% (26.3% mortality), 40% (10.5% mortality), and 20% (0% mortality) (Table 2). After 14d and 21d the overall percent mortality increased for all treatments (range = 4.5 – 100% and 39.1 – 100%, respectively), but the order of highest to lowest percent mortality remained the same (Table 2).

Adult Insecticide Trials. In 2011, there was a difference in adult hunting billbug mortality (Figure 6) among treatments ($P < 0.0001$). Bifenthrin (89.5%), indoxacarb (85%), chlothianidin (83.3%), and HGW (82.2%) all produced mortality >80%, while imidacloprid (69.2%) and chlorantraniliprole (19.2%) did not. There was low mortality in the controls (3.75%) In 2012, there was a difference in adult hunting billbug mortality (Figure 7) among treatments ($P<0.0001$). Bifenthrin + imidacloprid (85.4%) and chlothianidin (82.6%) all produced mortality >80%, while bifenthrin (66.6%), imidacloprid (65.6%), bifenthrin + Chlothianidin (61.8%), and dinotefuran (34.6%) did not. There was no mortality in the controls.

Larval Insecticide Trials. In 2012, there was a difference in larval hunting billbug mortality (Figure 8) among treatments ($P = 0.0005$). However, overall mortality was low. Imidacloprid (33.6%) had the highest mortality followed by chlothianidin (29.7%), bifenthrin (19.5%), and chlorantraniliprole (9.7%). There was no mortality in the controls.

Discussion

In North Carolina warm-season turfgrass, hunting billbugs overwinter as adults and small, large, and medium larvae; no pupae were found. The variation in life stages present indicates that when soil temperature decreases, the billbug will remain in its current life stage until ambient air temperatures increase. We were not able to determine if spring-collected billbugs had overwintered as adults or larvae. However once the adults were active in the spring (when approximate air and soil temperatures reach 10ºC in the spring; based on weather data during the first adult hunting billbug collection each Mar), they oviposited viable eggs. The absence of differences in the number of eggs oviposited each month and among years indicates that once the adult hunting billbugs are active they will oviposit until death or temperatures become too cold (when approximate air and soil temperatures reach 17 ºC in the fall; based on weather data during the last adult hunting billbug collection each Oct). The absence of differences in percent hatch and in general for number of eggs in reproductive tracts also indicates that once active, adults oviposit viable eggs. The differences among months for number of eggs in reproductive tracts could be an artifact of how many total females were collected at each date. Mar only had one female collected and the relatively wet spring and fall reduced the number of opportunities to collect females. The

decrease in number of eggs in the spring and fall could also indicate that the females had overwintered (when collected in spring) or were preparing to over winter (when collected in fall). Given the difficulty of determining time from eclosion of collected adults, it was not possible to determine the age of each adult beetle or how many generations were present during a calendar year. These data support previous findings of overlapping generations (Doskocil and Brandenburg 2012) of hunting billbugs in North Carolina.

Larvae survived best at soil moisture levels where 20% of the pores were occupied by water and mortality increased as more pores were filled with water. The higher soil moisture levels selected in this study represent moisture levels after moderate to heavy rainfall or irrigation events. The lower soil moisture levels represent moisture levels when rain events are less frequent. Given that not all areas in the turfgrass landscape have uniform soil moisture, mortality due to soil moisture will vary. If there are fewer rain events (where < 20% of total pores occupied by water), there could be an increase in larval mortality. An increase in larval mortality would subsequently decrease the number of emerging adults and could delay typical peak adult abundance. If there are frequent rain events (where > 20% of total pores occupied by water) and the soil remains at these levels for greater then seven days, larval mortality could also increase. Larval survival is also dependent on where the adults oviposit and how easily the larvae can move vertically in the soil to areas of optimal soil moisture. This could alter the depth at which larvae are located as well as their likelihood of survival to adulthood.

There were multiple products that provide mortality of hunting billbug adults. These results only include percent mortality 7 DAT. Preliminary experiments that assessed

mortality 14 DAT were not completed due to increased temperature in the containers that damaged the turfgrass. There were differences in the level of mortality for the products used to control hunting billbug larvae, however overall mortality was low (15-33.6%) indicating that the larger larvae are more difficult to control. This could be due to their vertical mobility within the soil profile; perhaps their larger size made the insect hardier, thus more difficult to kill. Larvae were collected from 7.6-22.8 cm in the soil profile. They were placed just below the turfgrass surface in these trials, but due to their large size, were able to move deeper in the soil profile.

We observed that hunting billbugs overwinter as adults and all larval stages, and once adults were active in the spring, they oviposit viable eggs. Soil moisture plays a role in larval survival and could alter the timing of adult emergence in the spring or fall. Vertical mobility of the large larvae makes this life stage more difficult to control. Monitoring adult populations yearly will be the key to effective management of the hunting billbug. Monitoring adults should involve several pitfall traps through the turfgrass area (Silcox et al. 2013), and checking these traps weekly to determine when the adults are active. If treatment is necessary, there are several classes of chemistry that can be chosen for effective control and should be applied at the peak of adult activity. These research findings on the hunting billbug biology and ecology in warm-season turfgrass provide additional information useful for developing a more precise management plan for this pest.

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Tables

Table 1. Total number and location of overwintering hunting billbug life stages in a bermudagrass stand from 2011-2013 in Raleigh, NC.

	Turfgrass and Soil				Soil Surface			
Year	Adult	Small Larvae	Medium Larvae	Large Larvae	Adult	Small Larvae	Medium Larvae	Large Larvae
2011	5	1	3	8	2	0	3	0
2012	8	0	1	2	0	1	1	0
2013	9	0	0	0	0	0	0	0

Table 2. Average percent mortality at 7, 14, and 21 days after introduction (DAI) of hunting billbug larvae held in containers maintained at soil moisture levels of 80, 60, 40, and 20 percent of total porosity.

Soil Moisture (%)[1]	7 DAI[2]	14 DAI	21 DAI
80	63.1 a	100 a	100 a
60	26.3 b	63.1 b	84 b
40	10.5 b	31.5 b	73.6 b
20	0 c	4.5 c	39.1 c

[1] Soil moisture levels based on percent of total pores occupied by water

[2]Data were analyzed using proc Glimmix and means were separated using least squares means ($F = 17.85$, df = 153, $P < 0.0001$). Means followed by the same letter are not different ($P < 0.05$).

Figures

Figure 1. Illustrations of container, food, and water source used to monitor oviposition behavior of female adult hunting billbugs collected in Raleigh, NC.

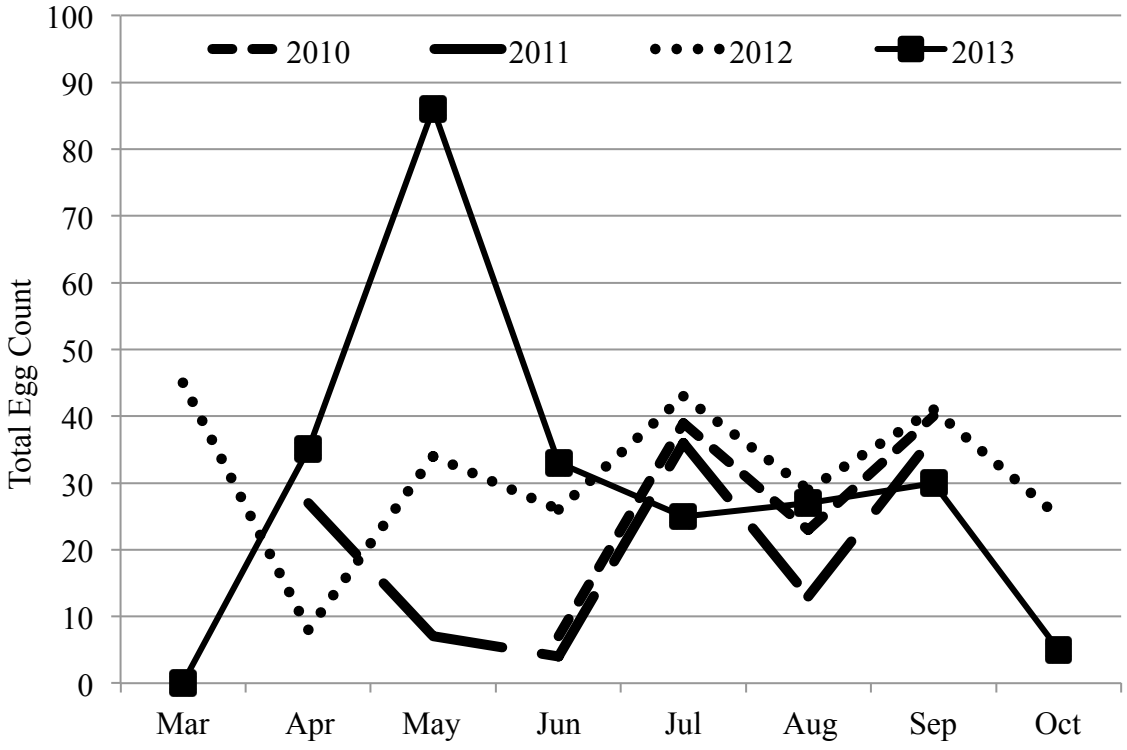


Figure 2. Total number of eggs oviposited per month by female adult hunting billbugs collected from 2010-2013, Raleigh, NC.

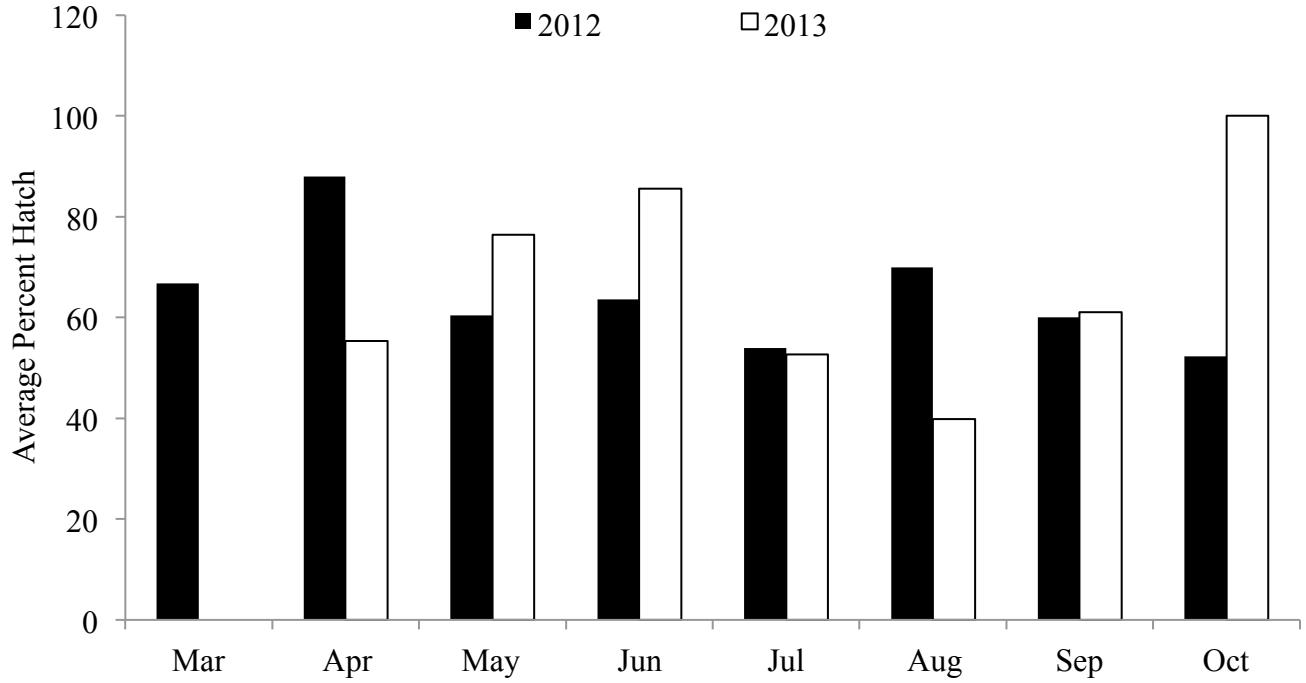
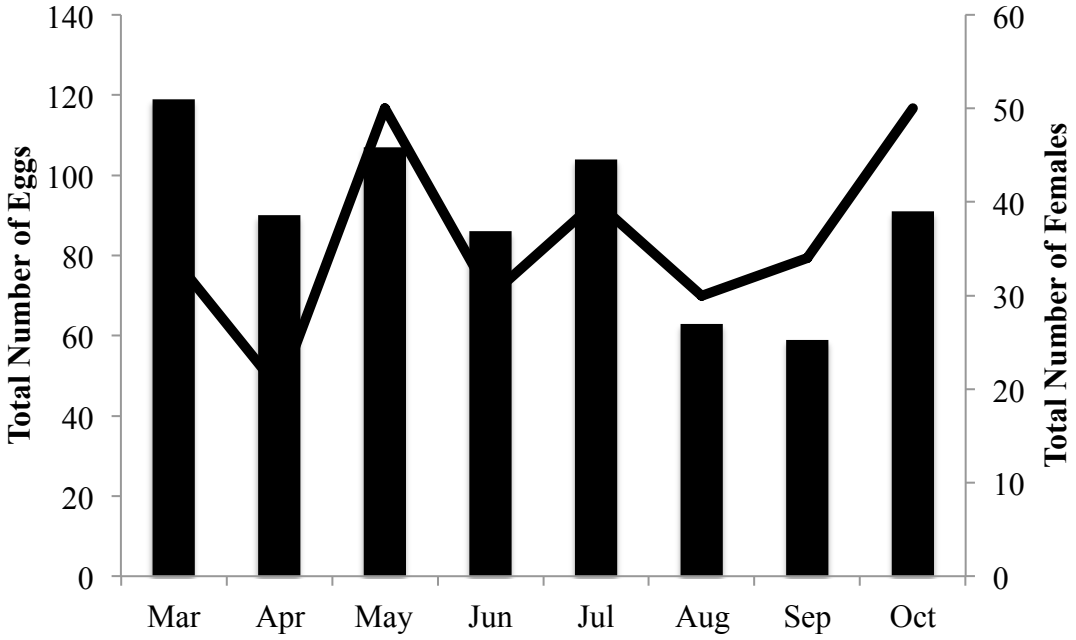


Figure 3. Average percent hatch for eggs oviposited per month by adult female hunting billbugs collected from 2012-2013, Raleigh, NC.



F**igure 4.** Total egg count remaining in reproductive tracts dissected from female adult hunting billbugs after being held in 30 ml containers for seven days per month in 2012, Raleigh, NC.

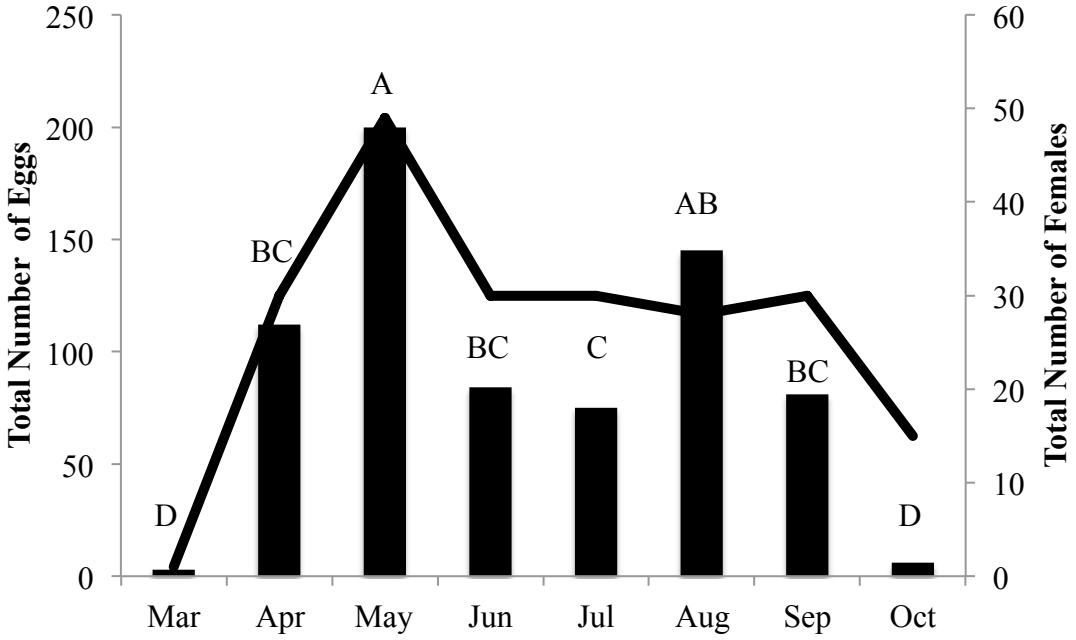


Figure 5. Total egg count remaining in reproductive tracts dissected from female adult hunting billbugs after being held in 30 ml containers for seven days per month in 2013, Raleigh, NC. Total number of eggs was analyzed using proc GLM and means were separated using Fisher's Protected LSD test ($F = 12.07$ df = 7, $P < 0.0001$). Means followed by the same letter are not different ($P<0.05$).

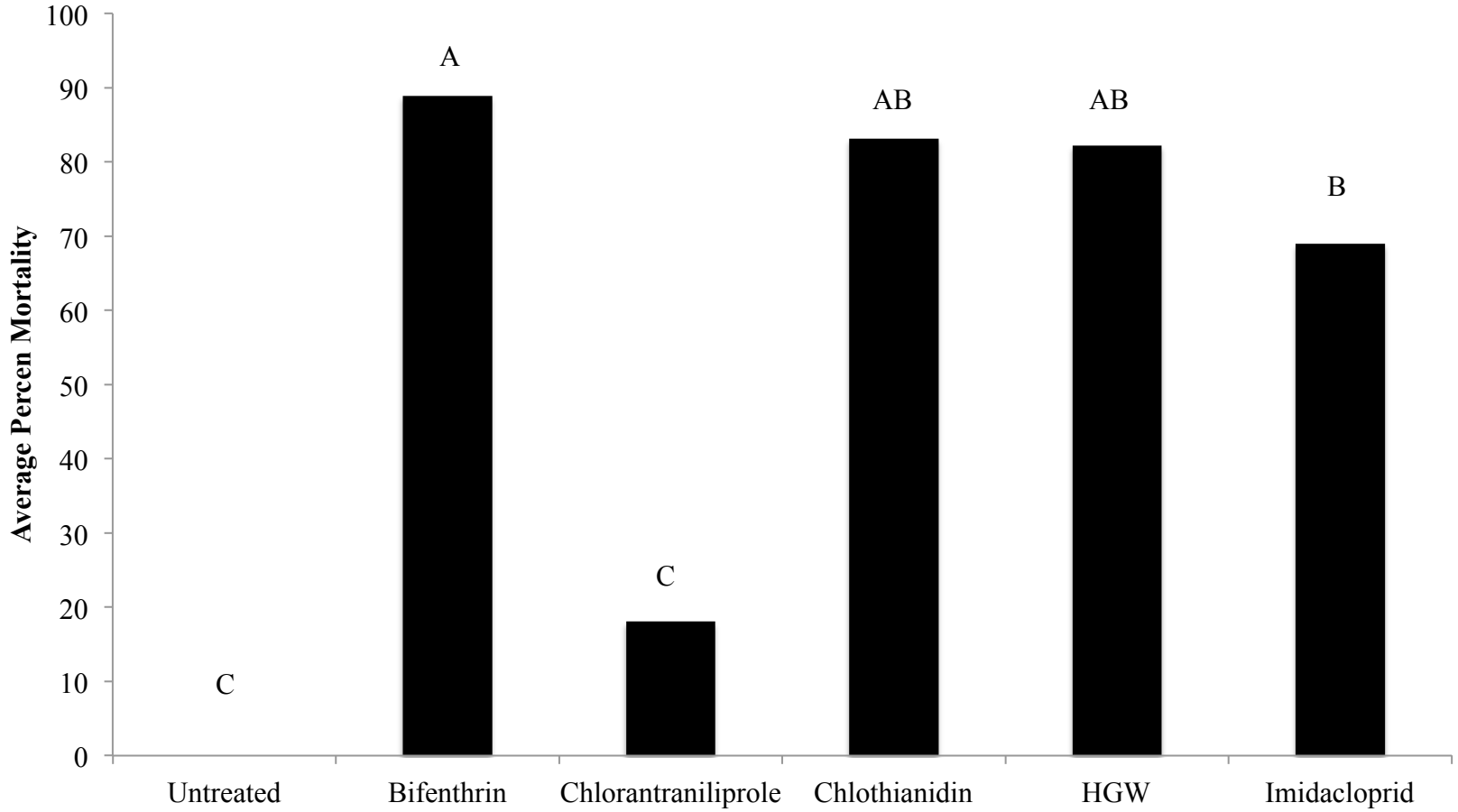


Figure 6. Average percent mortality of adult hunting billbugs 7 DAT in 2011. Data were analyzed using proc GLM and means were separated using Fisher's Protected LSD test ($F = 38.65$, df = 6, $P < 0.0001$). Means followed by the same letter are not different ($P<0.05$).

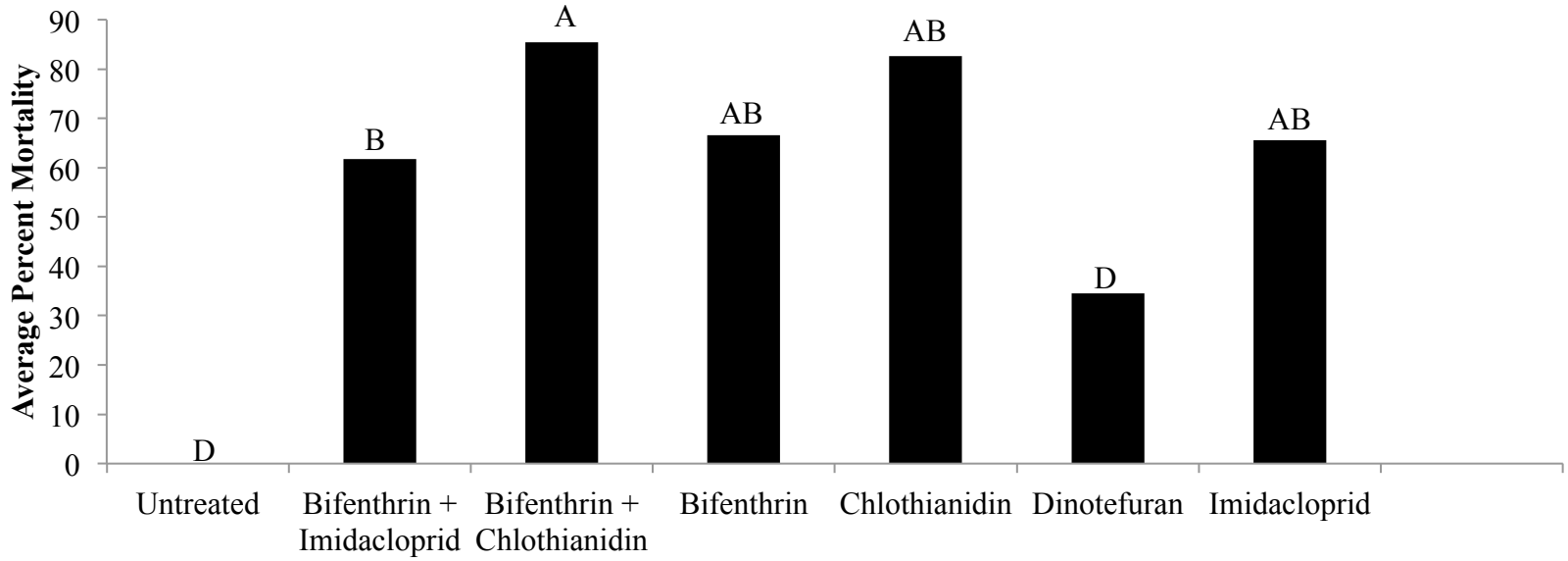


Figure 7. Average percent mortality of adult hunting billbugs 7 DAT in 2012. Data were analyzed using proc GLM and means were separated using Fisher's Protected LSD test ($F = 13.76$, df = 6, $P < 0.0001$). Means followed by the same letter are not different ($P < 0.05$).

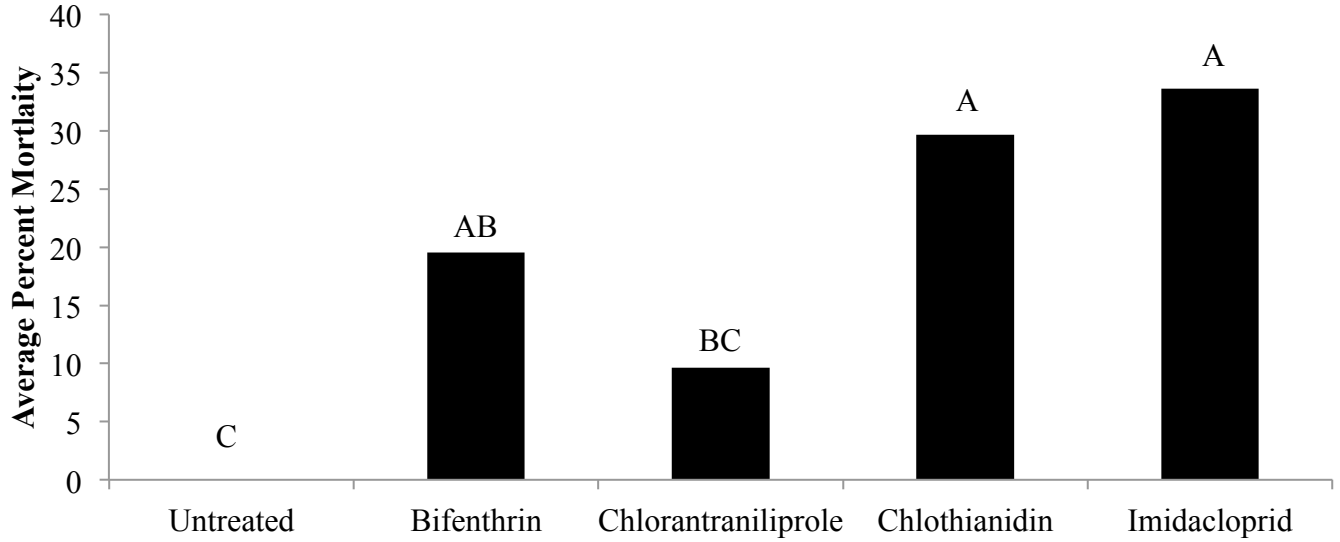


Figure 8. Average percent mortality of larval hunting billbugs 7 DAT in 2012. Data were analyzed using proc GLM and means were separated using Fisher's Protected LSD test ($F = 5.81$, df = 4, $P = 0.0005$). Means followed by the same letter are not different ($P < 0.05$).

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Using Goat Immunoglobulin to Determine Insect Feeding Behavior in Turfgrass

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Abstract

As new insecticide chemistries enter the market, further research is often necessary to determine efficacy and application timing on turfgrass insect pests. This is especially true for systemic insecticides that must be ingested by the insect to receive a lethal dose. Additionally, the mechanisms resulting in differences in control of adult hunting billbugs with systemic insecticides in cool- versus warm-season turfgrass require additional research on billbug feeding behavior. An enzyme-linked immunosorbent assay was developed using purified goat immunoglobulin (IgG) to treat specific portions of the turfgrass plant to determine billbug feeding behavior in warm- and cool-season turfgrass. The goat IgG was not translocated within the plant or leached into the soil, was able to be washed from the hunting billbug exoskeleton, and was detected in the hunting billbug gut after 12 hours of exposure. This technique may allow for future research on adult hunting billbug feeding behavior throughout a growing season. Information obtained with this technique may aid in developing a pest management plan that utilizes the most effective insecticides for targeting the specific billbug life stage present and for determining appropriate timing of the application based on billbug behavior.

Keywords *Sphenophorus venatus vestitus*, hunting billbug, enzyme-linked immunosorbent assay, bermudagrass, feeding behavior

Turfgrass systems are damaged by numerous insect pests each year. Managing these insects requires extensive knowledge of pest biology, ecology, effective insecticides, and application timing (Brandenburg and Freeman 2012). Legislative focus on reducing the environmental footprint of turf has forced many turfgrass managers to change their management programs for turfgrass insect pests (Gelernter 2012). Regulatory actions and the development of insecticide resistance, were two main factors influencing the agrichemical industry to develop new classes of chemistry that are effective and environmentally safer than previous products (Gelernter and Lomer 2000). As insect management strategies change, with new insecticides registered and older insecticide chemistries phased out, further studies are needed to confirm efficacy and application timing of insecticides for specific pests in turfgrass systems. This is especially important for systemic insecticides, which must be ingested by the insect for it to receive a lethal dose.

Adult hunting billbugs, *Sphenophorus venatus vestitus*, are small (8-11 mm) hard-bodied, black to deep red weevils (Vaurie 1951). The characteristic snout with chewing mouthparts on the tip and antennae inserted at the base of the snout help to identify them from other small beetles. In cool-season turfgrass, billbug larvae traditionally cause damage as a complex of five billbug species (Johnson-Cicalese 1988). It has been well documented that the larvae are responsible for the primary damage to cool-season turfgrass through feeding within stems and crowns of the plant. The adults cause minimal damage to turfgrass through oviposition scars (Vittum et al. 1999). In warm-season turfgrass, damage is primarily caused by one species of billbug, the hunting billbug (Doskocil 2010). Recent studies have shown that the adults cause the majority of damage to turfgrass (Doskocil and Brandenburg

2012). Larvae have been found to cause minimal to no damage in warm-season turfgrass (Doskocil and Brandenburg 2012).

Insecticide trials evaluating efficacy against hunting billbug in cool-season turfgrass identified excellent control with contact and systemic insecticides (Heller et al. 2007a, Heller et al. 2007b, Heller et al. 2008). In these trials, applications were timed to adult activity and assessed efficacy based on number of larvae present. Insecticide trials against hunting billbug adults in warm-season turfgrass produced excellent control with contact insecticides and poor control with systemic insecticides (Reynolds et al. 2014). These trials timed applications with adult activity and assessed efficacy based on number of adults present. Additional studies with systemic insecticides at different application timings also produced poor control of adult hunting billbugs (D.S. Reynolds, unpublished data). The application timing intervals were selected to allow time for the product to be translocated within the plant.

The difference in efficacy of systemic products against billbugs in cool- versus warm-season turfgrass could be related to the location of the pesticide in the plant relative to the feeding behavior and feeding site of these insects in the respective turfgrass types. The larvae feeding inside the stem of cool-season turfgrasses provides direct exposure to the systemic insecticides. The adult feeding behavior in warm-season turfgrasses has yet to be determined. The objective of this research was to develop a technique to help elucidate the feeding behavior of adult hunting billbugs in warm-season turfgrass.

Materials and Methods

Coating the ELISA Plate. The wells of a 96-well microplate (Product # 3590, Corning Life Sciences, Pittston, PA) were coated with 100 µl per well of rabbit anti-goat IgG

(ImmunoReagents, Inc., Raleigh, NC). After 60 min incubation at 25ºC, the rabbit anti-goat IgG was discarded and the plate rinsed three times with phosphate buffered saline (PBS)-Tween 20 (0.05% Tween) (Fisher Scientific, Fair Lawn, NJ). The plate was then blocked with 375 µl per well of PBS-Tween 20 and normal rabbit serum (ImmunoReagents, Inc., Raleigh, NC). After 60 min incubation at 25ºC, the solution was discarded and the plates were air dried for 30 min, wrapped in parafilm (Bemis Company, Inc., Neenah, WI) and placed in a refrigerator at 2°C until needed.

ELISA Protocol. Each 96-well plate included the following controls: (1) negative control for purified goat IgG, (2) 5 positive controls ranging from 2.5 – 0.15 ng purified goat IgG/ml, (3) PBS blanks, and (4) negative treatment controls. To prepare treatment samples, the material to be tested was submerged in1 ml of PBS per 1.5 ml centrifuge tube. The samples were incubated for 3 min at 25ºC then a 100 µl aliquot from each centrifuge tube was placed into individual wells. The samples were incubated for 60 min at 25ºC then discarded, the plate was rinsed three times with PBS-Tween 20, and then 100 µl of rabbit anti-goat IgG (H&L) biotin conjugate (ImmunoReagents, Inc., Raleigh, NC) was added to each well. The biotin was incubated for 60 min at 25ºC then discarded, the plate was rinsed five times with PBS-Tween 20, and 100 µl of alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) was added to each well. The streptavidin was incubated for 60 min at 25ºC, then discarded, the plate was rinsed five times with PBS-Tween 20, and 100 µl 1-step PNPP (Thermo Scientific, Rockford, IL) was added to each well. The PNPP was incubated for 10 min at 25ºC, then the absorbance of each well was measured using a ThermoMax microplate reader (Molecular Devices, LLC, Sunnyvale, CA)

set at a wavelength of 405 nm. Treatment samples were scored positive for the presence of purified goat IgG if they yielded an ELISA response four-standard deviations above the negative control mean (Sutula et al. 1986). The ELISA protocol was developed by ImmunoReagents, Inc., Raleigh, NC.

Proof of Concept Trials. To prepare the turfgrass before the ELISA, a core (10.16 cm in dia. X 10.16 cm depth) was removed from a bermudagrass stand in Raleigh, NC, and the soil was washed from roots. The core was divided in half; one half was used as the untreated plant, while the other half served as the treated plant. The roots or shoots of the untreated plant were submerged in 50 ml of phosphate buffered saline (PBS) (Sigma-Aldrich® Co. LLC, St. Louis, MO) (Figure 1), placed on a wire rack, treated side down, and allowed to air dry for 1 h (Figure 2). The roots or shoots of the treated plant were submerged in a solution of 50 ml PBS and 50 µl purified goat IgG (ImmunoReagents, Inc., Raleigh, NC)(Figure 1), placed on a wire rack, treated side down, and allowed to air dry for 1 h (Figure 2). After the purified goat IgG was dried on the leaf blades or roots, the plants were planted in a small plastic cup (Member's Mark clear plastic cups, 266 ml, Bentonville, AR) using Profile Porous Ceramic Greens Grade soil amendment (Profile Products LLC, Buffalo Grove, IL) and irrigated with75 ml of water (Figure 3).

Translocation. To determine if the purified goat IgG was translocated within the plant, bermudagrass plant shoots were treated as described above. After 12 h, treated shoot material, untreated root material (from a plant with treated shoots) and untreated soil (from plant with treated shoots) was removed and placed in 1.5 ml micro centrifuge. Approximately 0.1 g of plant or soil material was removed by scissor for plant material and a

metal laboratory spatula for soil material. A second trial included bermudagrass treated roots, untreated shoots (from a plant with treated roots), and untreated soil (from plant with treated roots). In both trials untreated shoot, root, and soil material were used as a negative control. The turfgrass and soil samples were placed individually in 1.5 ml micro centrifuge tubes and the ELISA proceeded as described previously.

Washing. To determine that no purified goat IgG remained on the insect exoskeleton several washing techniques were tested. Adult hunting billbugs were collected by hand at night from a bermudagrass stand and held for 12 h in plastic deli cups (950 ml, Tripak Industrial USA, LLC, White Plains, NY) with moistened paper towels. After starving the beetles for 12 h they were placed in a 50 ml centrifuge tube (Grainger®, Raleigh, NC), and put in the freezer for 20 min at -17°C. After 20 min the billbugs were taken out of the freezer, and individually treated with a solution of 50 ml PBS and 50 µl purified goat IgG. The treatment solution was applied by dipping a cotton swab (Johnson & Johnson, New Brunswick, NJ) in the solution, then rubbing the treated cotton swab over the elytra of each beetle. The beetles were placed dorsal side up on a paper towel to dry for 5 min. Washing treatments included: three min ultrasonic bath in PBS and rinsed three times with PBS; three min ultrasonic bath with PBS-Tween 20 and rinsed three times with PBS-Tween 20; three min ultrasonic bath in PBS and rinsed three times with PBS-Tween 20; and thirty min ultrasonic bath in 100 ml PBS + 0.1g trypsin (Product Code T6567, Sigma®, St. Louis, Missouri) and rinsed three times with PBS-Tween 20. Untreated beetles and treated, unwashed beetles were included as negative and positive controls, respectively. The beetles

were placed individually in 1.5 ml micro centrifuge tubes and the ELISA proceeded as described previously.

Beetle feeding. Adult hunting billbugs were collected by hand at night from a bermudagrass turfgrass surface in Raleigh, NC and placed on the soil surface in either a container planted with treated or untreated turfgrass (Figure 4). Turfgrass treatments were applied as described above. After 12 h the billbugs were removed from the turfgrass plug, placed in a 50 ml centrifuge tube, and put in the freezer for 20 min at -17°C. After 20 min the billbugs were taken out of the freezer, removed from the centrifuge tube and placed in an ultra-sonic cleaner (CD-2800, Interteck Group, Wilmington, NC) that contained a solution of 100 ml PBS and 0.1 g of trypsin (Product Code T6567, Sigma®, St. Louis, Missouri) for 30 min. The beetles were placed in a 50 ml centrifuge tubes and rinsed three times with PBS-Tween 20. The beetles were then placed individually in 1.5 ml micro centrifuge tube (Grainger®, Raleigh, NC) and the bodies were cut in quarters using a pair of dissecting scissors (American Educational Products, Fort Collins, CO). Treatments consisted of beetles that fed on untreated bermudagrass shoots or roots and were not washed and beetles that fed on treated bermudagrass shoots or roots and were washed.

Results

Proof of Concept Trials. *Translocation*. There was no translocation of purified goat IgG in the bermudagrass plant when either the roots or shoots were treated (Table 1). There was also no leaching of purified goat IgG into the soil (Table 1).

Washing. The untreated beetles all tested negative for the presence of purified goat IgG (Table 2). The treated beetles all tested positive for the presence of purified goat IgG. For

treated beetles place in an ultrasonic bath and rinsed with either PBS or PBS-Tween 20, 66% tested positive for the presence of purified goat IgG. For treated beetles placed in an ultrasonic bath with PBS and rinsed with PBS-Tween 20, 33% tested positive for the presence of purified goat IgG. For treated beetles placed in an ultrasonic bath with trypsin and rinsed with PBS-Tween 20 all tested negative for the presence of purified goat IgG.

Beetle feeding. The beetles exposed to untreated shoot material all tested negative for the presence of purified goat IgG (Table 3). Of the beetles exposed to treated shoot material, 83% tested positive for the presence of purified goat IgG. The beetles exposed to both untreated and treated root materials all tested negative for the presence of purified goat IgG.

Discussion

Developing economical and environmentally conscious pest management strategies require current information on pest biology and behavior. Understanding the hunting billbug feeding behavior is necessary for selecting the appropriate insecticide and applying the insecticide when the adults are active. Applying the insecticide at the appropriate time will result in maximum efficacy with the insecticide chosen and reduce environmental input. The ELISA technique described in this paper allowed determination of which part of the turfgrass plant adult hunting billbug feed on without translocation of the target protein to other parts of the plant or leaching into the soil profile. This protein can be removed from the insect exoskeleton and can be detected in the insect gut. Adult hunting billbugs feed on shoot material of bermudagrass. Not all beetles exposed to bermudagrass treated with purified goat IgG tested positive. This could indicate that adult hunting billbugs do not feed consistently over the exposure time used in these experiments. It could also indicate that there were

undetectable amounts remaining in the insect gut. The assay is able to detect the purified goat IgG as low as 0.15 ng IgG/ml; however, if the goat IgG is digested prior to testing, it would not be detected. Future studies should include investigations into the effects of time on adult hunting billbug digestion of the purified goat IgG to determine the optimal testing interval.

When managing the hunting billbug in warm-season turfgrass, managers should target insecticide applications to periods when adults are at peak abundance to achieve maximum efficacy with minimal environmental input. This requires monitoring with pitfall traps or scouting the turfgrass surface at night with a flashlight (Silcox et al. 2013), mainly in the spring and fall when adults are known to be most active and most abundant. When using a systemic insecticide, applications should be made with sufficient time for the material to be translocated within the plant to a site of adult billbug feeding and when adult billbugs are feeding. Also, the insecticide or its metabolites should remain in the shoot material long enough for the adult billbugs to feed and receive a lethal dose. Continued research on adult hunting billbug feeding behavior should focus on the effect of time on purified goat IgG digestion. Once the optimum timing is determined, experiments should focus on their feeding behavior throughout the growing season. Knowing when adult hunting billbugs feed will allow for the proper selection and application timing of insecticide applications.

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Tables

Table 1. ELISA optical density range and critical values for testing to determine if purified goat IgG when applied to either shoots or roots of bermudagrass, treated at 50 µl goat IgG in 50 ml phosphate buffered saline, is translocated to the rest of the turfgrass plant or leached into the soil. Untreated shoots or roots of bermudagrass were tested to determine the baseline optical density readings.

Treatment	ELISA Optical Density Range	Critical Value $\bar{x}$ ± 4 SD	Treatment	ELISA Optical Density Range	Critical Value $\bar{x}$ ± 4 SD
Trt Shoots	1.33-2.93	0.291	Untrt Shoots	0.20-0.27	0.291
Roots	0.19-0.26	0.291	Roots	0.18-0.22	0.291
Soil	0.18-0.21	0.291	Soil	0.17-0.20	0.291
Trt Roots	2.93-2.27	0.247	Untrt Roots	0.15-0.17	0.247
Shoots	0.16-0.18	0.247	Shoots	0.15-.017	0.247
Soil	0.15-0.20	0.247	Soil	0.15-0.16	0.247

Table 2. ELISA optical density range, critical values, and percent testing positive for determining a washing technique when adult hunting billbug elytra were treated with purified goat IgG at 50 µl goat IgG in 50 ml phosphate buffered saline.

Treatment	ELISA Optical Density Range	Critical Value $\bar{x}$ ± 4 SD	Percent Positive
Untreated/ Unwashed	0.21-0.24	0.285	0
Treated/ Unwashed	1.62-2.91	0.285	100
Ultrasonic bath w/ PBS Rinse w/ PBS	0.24-0.37	0.285	66
Ultrasonic bath w/ PBS-Tween 20 Rinse w/ PBS-Tween 20	0.27-0.38	0.285	66
Ultrasonic bath w/ PBS Rinse w/ PBS Tween-20	0.23-0.47	0.285	33
Ultrasonic bath with Trypsin Wash with PBS Tween-20	0.18-0.21	0.285	0

Table 3. ELISA optical density range, critical value, and percent testing positive for testing of adult hunting billbugs that were exposed to either bermudagrass shoots or roots treated with purified goat IgG at 50 µl goat IgG in 50 ml phosphate buffered saline.

Treatment	ELISA Optical Density Range	Critical Value $\bar{x}$ ± 4 SD	Percent Positive
Untreated Shoots	0.08-0.12	0.161	0
Treated Shoots	0.15-0.43	0.161	83
Untreated Roots	0.52-2.84	3.77	0
Treated Roots	1.06-2.98	3.77	0

Figures

Figure 1. To treat the shoots of the turfgrass plant, the excess soil was removed from the roots, the turfgrass core was inverted and the shoots were submerged in 50 ml PBS with 50 µl purified goat IgG to coat the shoots.

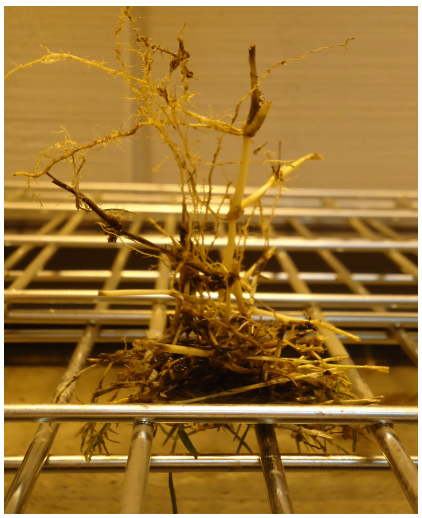

Figure 2. After the turfgrass shoots were coated with purified goat IgG solution the core was placed treated part down on a wire rack to air dry for one hour.

Figure 3. The treated and dried turfgrass plant was replanted in a small plastic cup using Profile Porous Ceramic Greens grade soil amendment and irrigated with75 ml of water.

Figure 4. Adult hunting billbugs were collected at night by hand and placed on the soil surface of the treated or control plant container.

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Feeding Behavior of Adult Hunting Billbugs (Coleoptera: Curculionidae) in North Carolina

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Abstract

The incidence of hunting billbug activity in turfgrass transition zones such as North Carolina has increased since 2000. Effective management of this pest requires information on its feeding behavior. Damage assessment and feeding behavior trials were developed to determine the damage threshold of hunting billbug adults and on what part of the turfgrass plant adult hunting billbugs are feeding. Containers were planted with bermudagrass, zoysiagrass, or tall fescue and billbug treatments consisted of 0, 2, 4, or 6 adult hunting billbugs. Photographs of the turfgrass surface were taken prior to billbug introduction, and then every 7 d for four weeks after billbug introduction, and analyzed for percent cover. An enzyme-linked immunosorbent assay (ELISA) was developed to mark either the shoots or roots of the turfgrass plant with purified goat immunoglobulin (IgG). Adult hunting billbugs were introduced into the containers with IgG treated turfgrass. After 12 h of exposure to the treated turfgrass the billbug guts were tested for the presence or absence of purified goat IgG. Overall, the containers that had 0 billbugs had the highest average percent green cover. There were no differences in average percent green cover among photograph dates. The ELISA technique provided data indicating that billbugs feed on warm-season turfgrass stems, but only a low percentage actually tested positive. These results indicate that adult hunting billbugs reduce average percent green cover in warm-season turfgrass.

Keywords *Sphenophorus venatus vestitus*, hunting billbug, damage assessment, feeding behavior

There are over 60 billbug (Coleoptera: Curculionidae) species in the United States (Niemczyk and Shetlar 2000) and nine of those species are known to feed on turfgrass in the United States (Vittum et al. 1999). In general, these billbugs are hard-bodied, black to deep red weevils that vary in length from 8 to 11 mm (Vaurie 1951). Adult billbugs have a characteristic snout with chewing mouthparts on the tip. The larvae are creamy white with a brown head capsule and reach a length of about 9.5 mm at maturity (Potter 1998). In cool-season turfgrass there is a complex of five billbug species that cause visible damage (Johnson-Cicalese 1988). It has been well documented in cool-season turfgrass that the adults cause minimal damage to the turfgrass through oviposition scarring and feeding. In these systems the larvae cause the majority of damage as they feed within the turfgrass stem. When feeding, the larvae hollow out the stem of the turfgrass, moving downwards towards the crown of the plant. When they become too large for the stem, they will feed on the crown of the plant, then drop into the soil and feed on the roots (Vittum et al. 1999).

In warm-season turfgrass, there are four billbug species that cause visible damage; however two species, *Sphenophorus coesifrons* and *S. apicalis*, are sporadic in their distribution and damage potential (Vittum et al. 1999). The Phoenician billbug *S. phoeniciensis* is limited to southern California and Arizona, where it is the most common billbug species. The hunting billbug, *S.venatus vestitus* Chittenden, is the most common billbug in warm-season turfgrass, primarily in the southern United States (Shetlar et al. 2012). Preliminary studies in warm-season turfgrass indicate the adults are the most damaging life stage, while the larvae cause little to no visible damage (Doskocil and Brandenburg 2012). There have been no studies conducted to determine if the damage seen

in warm-season turfgrass from adult billbugs is a result of feeding on the turfgrass stems, feeding on the turfgrass roots, or disturbance of the soil, with consequent damage to the root system. Furthermore, there have been no comparisons of damage caused by the hunting billbug in warm vs. cool-season turfgrass. In transition zones (areas that grow both warm and cool-season turfgrass), such as North Carolina, it is important to understand the damage threshold of this insect in both turfgrass types.

The development of an effective management plan for hunting billbugs in North Carolina and other transition zones would be enhanced with the availability of more information about adult hunting billbug feeding behavior. The objectives of this research were to determine the level of damage produced by adult hunting billbugs in warm and cool-season turfgrass, and to determine on what part of the turfgrass plant the adult hunting billbugs are feeding.

Materials and Methods

Damage Assessment Trials. Adult hunting billbug injury was determined in warm and cool-season turfgrasses through the use of digital imaging analysis. This method allows for estimation of billbug injury based on percent green turfgrass cover. *Trial 1.* In 2010, thirty-two polyvinyl chloride pipes (15 cm dia x 20 cm length) were inserted into the ground using downward pressure from the bucket of a backhoe to a depth of 15 cm into established turfgrass stands at the Sandhills Research Station, Jackson Springs, NC. Sixteen PVC pipes were placed in bermudagrass, *Cynodon dactlyon x Cynodon transvaalensis*, and sixteen in zoysiagrass, *Zoysia japonica*. The turfgrass was irrigated three times per week for 10 min,

and was not mowed for the duration of the study. Billbug treatments applied to each grass species consisted of 0, 2, 4, or 6 billbugs in a 1:1 male to female ratio, with four replicates per treatment. Adult hunting billbugs were collected by hand from a bermudagrass surface at Raleigh Golf Association in Raleigh, NC and placed into plastic cups with moistened paper towels for transport. Within 24 h after collection they were introduced into the PVC enclosures, fiberglass screen was placed over the PVC pipes, and this was secured with a 0.91 m cable tie (Cable Ties Plus Inc., Kingston, MA). Billbugs were introduced into containers on 16 Jul 2010. Digital images were recorded weekly for four weeks using a Sony Cyber-Shot DSC-W530 camera with the lens placed in a 3.17 dia. hole on top of a cardboard light box (44.45 X 36.83 cm). One, 30.4 cm, white fluorescent battery operated light (Model No. 17406, General Electric®, Fairfield, CN) was attached to each of the four sides of the box with adhesive Velcro (Velcro®, Sticky Back®, Levitt Industrial Textile, Hicksville, NY) for a total of four lights. Adult hunting billbug injury in warm-season turfgrass was documented by analyzing digital images for percent green cover using Sigmascan Pro digital imaging software (SPSS Inc., Chicago, IL) and the Sigmascan Pro macro named "Turf Analysis" (Karcher and Richardson, 2005). The macro calculates percent cover by determining the number of green pixels in the photograph and dividing that number by the total number of pixels in the photograph. Green pixels were defined using hue (45-120) and saturation (20-100) values. This range of hue and saturation values were determined based on what most accurately represented green turf cover for these images. All data were subjected to ANOVA to determine treatment effects (Proc GLM, SAS Institute, Cary, NC). Treatments

were subjected to Fisher's Protected LSD test at the 0.05 probability level when *F*-test indicated significant treatment effects.

Trial 2. In 2012, three experiments (A, B, and C) consisted of ninety-six clear plastic deli containers (950 ml, Tripak Industrial USA, LLC, White Plains, NY) modified for use in adult hunting billbug damage assessment trials. Modifications included 5 drainage holes (0.5 cm diameter), made with a soldering iron in the bottom of the container and fiberglass screen (0.027cm mesh dia., New York Wire Company, Mount Wolf, PA) glued to the bottom to prevent insect escape. Forty-eight plugs each of bermudagrass and turf-type tall fescue were removed from a turfgrass stand using a standard golf course cup-cutter (10.16 cm in dia.) and transplanted into the containers. After planting, all containers were placed into a greenhouse at North Carolina State University Raleigh, NC one month prior to billbug introduction. The turfgrass was irrigated three times per week, mowed weekly at 3.81 cm for bermudagrass and 7.62 cm for tall fescue, and fertilized monthly using 20-20-20 Scotts Miracle-Gro®All-Purpose Plant Food (The Scotts Company, Marysville, OH). This irrigation and fertilization schedule remained through the duration of the experiment. Billbug treatments applied to each grass species consisted of 0, 2, 4, or 6 billbugs in a 1:1 male to female ratio, with four replicates per treatment. Adult hunting billbugs were collected by hand from a bermudagrass surface at Raleigh Golf Association in Raleigh, NC and placed into plastic cups with moistened paper towels. Within 24 h of collection, billbugs were placed into each container and covered with tulle fabric (Paper Mart®, Orange, CA), which was held in place by rubber bands to prevent insect escape during the study. Billbugs were introduced into containers for

experiments A, B, and C on 2 Oct 2012, 18 Oct 2012, and 26 Oct 2012 respectively. Digital images were recorded 1 h prior to billbug introduction and weekly for four weeks after introduction using a Canon EOS Rebel XS camera with a 50mm Cannon EF lens (Canon USA, Inc., Melville, NY) that was mounted to an aluminum light box (60.9 X 50.8 X 55.8 cm) with four fluorescent lights attached inside. Adult hunting billbug injury in warm and cool-season turfgrass was documented by analyzing digital images for percent green cover using the same methods described for trial 1. Green pixels were defined using hue (60 - 120) and saturation (20-100) values.

Trials 3 & 4. In 2013, three experiments (A, B, and C) in trial 3 and two experiments (A and B) in trial 4 consisted of containers in controlled-environment growth chambers at the Southeastern Plant Environment Laboratory at North Carolina State University in Raleigh, NC. Ninety-six black square nursery containers (1,048 ml, Growers Supply, Dyersville, IA) were modified for use in adult hunting billbug damage assessment trials. Modification consisted of a piece of fiberglass screen (0.027cm mesh dia., New York Wire Company, Mount Wolf, PA) glued to the bottom to prevent insect escape. Forty-eight plugs each of bermudagrass and turf-type tall fescue, *Festuca arundinacea*, were removed from a turfgrass stand using a standard golf course cup-cutter (10.16 cm in dia.) and transplanted into the containers. After planting, all containers were placed into growth chambers one month prior to billbug introduction. Bermudagrass treatments were maintained at day/night temperatures of 29/24°C, respectively, with a 10-hr photoperiod (0700 h to 1700 h). Tall fescue treatments were maintained at day/night temperatures of 26/22°C, respectively, with a 10-hr

photoperiod (0700 h to 1700 h). Water and nutrient solution (NCSU, 2011) were applied twice daily to the bermudagrass containers and a nutrient solution was applied once daily for the tall fescue containers. Billbug treatments applied to each grass species consisted of 0, 2, 4, or 6 billbugs in a 1:1 male to female ratio, with four replicates per treatment. Adult hunting billbugs were collected by hand from a bermudagrass surface at Raleigh Golf Association in Raleigh, NC and placed into plastic cups with moistened paper towels. Within 24 h after the billbugs were collected, all grass was mowed and the billbugs were immediately placed into each container and then covered with tulle fabric, which was held in place by rubber bands to prevent escape during the study. Billbugs were introduced into containers for trial 3, experiments A, B, and C on 25 Apr 2013, 6 May 2013, 15 May 2013, respectively and for trial 4, experiments A and B on 16 Aug 2013, and 23 Aug 2013, respectively. Digital images were recorded 1 h prior to billbug introduction and weekly for four weeks after introduction. Digital images were analyzed using the same methods as described for trial 1 using the same hue and saturation values as described in trial 2.

Feeding Behavior Trials. A sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine where on the turfgrass plant field-collected hunting billbugs feed. The technique and preliminary trials was described in detail by Billeisen et al. (2014). Briefly, the wells of a 96-well microplate (Product # 3590, Corning Life Sciences, Pittston, PA) were coated with 100 µl per well of rabbit anti-goat IgG (ImmunoReagents, Inc., Raleigh, NC). The rabbit anti-goat IgG was incubated for 60 min at 25ºC then discarded and the plate rinsed three times with phosphate buffered saline (PBS)-Tween 20 (0.05% Tween) (Fisher

Scientific, Fair Lawn, NJ). The plate was then blocked with 375 µl per well of PBS-Tween 20 and normal rabbit serum (ImmunoReagents, Inc., Raleigh, NC) solution. The solution was incubated for 60 min at 25ºC then was discarded and the plates were air dried for 30 min, wrapped in Parafilm® M (Bemis Company, Inc., Neenah, WI) and placed in a refrigerator at 2°C until needed.

To prepare the turfgrass prior to billbug introduction, a standard cup-cutter core of bermudagrass, zoysiagrass, or tall fescue was removed from the turfgrass stand and the soil was washed from roots. The core was divided in half; one half used as the untreated plant, the other half as the treated plant. The roots or shoots of the untreated plant were submerged in 50 ml of phosphate buffered saline (PBS) (Sigma-Aldrich® Co. LLC, St. Louis, MO) and placed on a wire rack, treated side down, and allowed to air dry for 1 h. The roots or shoots of the treated plant were submerged in a solution of 50 ml PBS and 50 µl purified goat immunoglobulin (IgG) (ImmunoReagents, Inc., Raleigh, NC) and placed on a wire rack, treated side down, and allowed to air dry for 1 h. After the marker dried on the plant blades or roots the plants were replanted in a small plastic cup (Member's Mark clear plastic cups, 266 ml, Bentonville, AR) using Profile Porous Ceramic Greens Grade soil amendment (Profile Products LLC, Buffalo Grove, IL) and irrigated with 75 ml of water. Adult hunting billbugs were collected by hand at night from a bermudagrass surface in Raleigh, NC. Billbugs were placed on the soil surface in either the treated or untreated container within 24 h of collection. After 12 h the billbugs were removed from the turfgrass plug, placed in a 50 ml centrifuge tube (Grainger®, Raleigh, NC), and put in the freezer for 20 min at -17°C.

After 20 min the billbugs were taken out of the freezer, removed from the centrifuge tube and placed in an ultra-sonic cleaner (CD-2800, Interteck Group, Wilmington, NC) that contained a solution of 100 ml PBS and 0.1 g of trypsin (Product Code T6567, Sigma®, St. Louis, Missouri). The beetles were in the ultrasonic bath for 30 min, placed in a 50 ml centrifuge tube, and rinsed three times with PBS-Tween 20. The beetles were then placed individually in 1.5 ml micro centrifuge tube (Grainger®, Raleigh, NC) and the insect bodies were cut in quarters using a pair of dissecting scissors (American Educational Products, Fort Collins, CO). The scissor blades were cleaned with 95% ethanol between the cutting of each beetle.

Each 96-well plate included the following controls: (1) negative control for purified goat IgG, (2) 5 positive controls ranging from 2.5 – 0.15 ng purified goat IgG/ml, (3) PBS blanks, and (4) negative adult hunting billbugs (i.e. beetles exposed to untreated turfgrass). To prepare the billbug samples, the dissected billbugs were submerged in1 ml of PBS per 1.5 ml centrifuge tube. The samples were incubated for 3 min at 25ºC then a 100 µl aliquot from each centrifuge tube was placed into individual wells. The samples were incubated for 60 min at 25ºC, then were discarded; the plate was rinsed three times with PBS-Tween 20, and 100 µl of rabbit anti-goat IgG (H&L) biotin conjugate (ImmunoReagents, Inc., Raleigh, NC) was added to each well. The biotin was incubated for 60 min at 25ºC then discarded, the plate was rinsed five times with PBS-Tween 20, and 100 µl of alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) was added to each well. The streptavidin was incubated for 60 min at 25ºC then discarded, the plate was rinsed five times with PBS-Tween 20, and 100 µl 1-step PNPP (Thermo Scientific, Rockford, IL) was added

to each well. The PNPP was incubated for 10 min at 25ºC, then the absorbance of each well was measured using a ThermoMax microplate reader (Molecular Devices, LLC, Sunnyvale, CA) set at a wavelength of 405 nm. Adult hunting billbugs were scored positive for the presence of purified goat IgG if they yielded an ELISA response three-standard deviations above the negative beetle control mean (Sutula et al. 1986).

Results

Damage Assessment Trials. The ANOVA (Table 1) indicated that there was a significant study and experiment effect and thus each study and each subsequent experiment were analyzed separately. *Trial 1.* The bermudagrass trials had differences in average percent green cover among billbug treatments (Table 2). The 0 billbug treatment had the highest average percent green cover followed by 6, 4, and 2 billbug treatments, respectively. There were differences in average percent green cover among photograph dates in the 0 and 6 billbug treatments, but not in the 2 and 4 billbug treatments (Table 3). The zoysiagrass trials had differences in average percent green cover among billbug treatments (Table 4). The 0 billbug treatment had the highest average percent green cover followed by 4, 6, and 2 billbug treatments, respectively. There were no differences in average percent green cover among photograph dates in any billbug treatment (Table 5). Overall there was no difference in percent cover between bermudagrass and zoysiagrass.

Trial 2. The bermudagrass trials had differences in average percent green cover among billbug treatments in all experiments (Table 6). In experiment A, the 0 billbug treatment had the highest average percent green cover followed by 4, 2, and 6 billbug treatments, respectively. In experiment B, the 0 billbug treatment had the highest average

percent green cover followed by 2, 4, and 6 billbugs, respectively. In experiment C, the 0 billbug treatment had the highest average percent green cover followed by 2, 6, and 4 billbugs, respectively. In experiment A, there were differences in average percent green cover among photograph dates for all billbug treatments (Table 7). In experiment B, there were no differences in average percent green cover among photograph dates for all billbug treatments (Table 8). In experiment C, there were differences in average percent green cover among photograph dates for the 0, 2, and 4 billbug treatments, but no differences for the 6 billbug treatment (Table 9).

The tall fescue trials had no differences in average percent green cover among billbug treatments (Table 10) in experiments A, B, or C. In experiment A, there were differences in average percent green cover among photograph dates for the 2 and 4 billbug treatments, but no differences for the 0 and 6 billbug treatments (Table 11). In experiment B, there were differences in average percent green cover among photograph dates for the 0, 2, and 6 billbug treatments, but no the 4 billbug treatment (Table 12). In experiment C, there were differences in average percent green cover among photograph dates for the 0 and 6 billbug treatments, but no differences for the 2 and 4 billbug treatments (Table 13).

Trial 3. The bermudagrass trials had differences in average percent green cover among billbug treatments for experiment C (Table 14). The 4 billbug treatment had the highest average percent green cover followed by 6, 2, and 0 billbug treatments, respectively. In experiments A (Table 15) and B (Table 16) there were no differences in average percent green cover among photograph dates for all billbug treatments. In experiment C, there was a

difference in average percent green cover among weeks for the 6 billbug treatment, but no differences for the 0, 2, or 4 billbug treatments (Table 17).

The tall fescue trials had no differences in average percent green cover among billbug treatments for all experiments (Table 18). In experiment A, there was a difference in average percent green cover among photograph dates for the 2 billbug treatment, but no differences for the 0, 4, or 6 billbug treatments (Table 19). In experiments B (Table 20) and C (Table 21) there were no differences in average percent green cover among photograph dates for all billbug treatments.

Trial 4. The bermudagrass trials had no difference in average percent green cover among billbug treatments for all experiments (Table 22). In experiment A, there was a difference in average percent green cover among photograph dates for the 4 billbug treatment, but no differences for the 0, 2, or 6 billbug treatments (Table 23). In experiment B, there were no differences in average percent green cover among photograph dates for all billbug treatments (Table 24).

The tall fescue trials had differences in average percent green cover among billbug treatments for both experiments (Table 25). In experiment A, the 6 billbug treatment had the highest average percent green cover followed by 4, 2, and 0 billbug treatments, respectively. In experiment B, the 0 billbug treatment had the highest average percent green cover followed by 6, 4, and 2 billbug treatments, respectively. In experiment A, there were no differences in average percent green cover among photograph dates for all billbug treatments (Table 26). In experiment B, there was a difference in average percent green cover among

photograph dates for the 0 billbug treatment, but no differences for the 2, 4, or 6 billbug treatments (Table 27).

Feeding Behavior Trials. Of the 12 billbugs exposed to treated bermudagrass shoot material, 6 (50%) tested positive for purified goat IgG (Table 28). Billbugs exposed to treated bermudagrass root material did not test positive for purified goat IgG. Of the 12 billbugs exposed to treated zoysiagrass shoot material, 1 (8%) tested positive for purified goat IgG (Table 28). Billbugs exposed to treated zoysiagrass root material did not test positive (Table 28) for purified goat IgG. Billbugs exposed to either treated tall fescue shoot or root material did not test positive (Table 28) for purified goat IgG.

Discussion

In general, in all trials where there were differences among billbug treatments; digital imaging analysis indicates that adult hunting billbugs reduced the percent green cover compared to the controls. These trials used a much lower number of billbugs (0, 2, 4, or 6) compared to previous studies (0, 6, 10, or 16) (Doskocil and Brandenburg 2012), which indicates that even a moderate billbug population can reduce turfgrass cover. In the experiments with warm-season turfgrass, 60% had differences among billbug treatments, while in the experiments with cool-season turfgrass, 12% had differences among billbug treatments. This is consistent with previous studies that have found adult hunting billbugs to reduce the greenness and height of warm-season turfgrass (Doskocil and Brandenburg 2012), but cause minimal damage in cool-season turfgrass (Vittum et al. 1999). The data did not indicate a trend in average percent green cover among photograph dates for any billbug treatment for all studies and experiments within studies. Only 23% of the photograph dates

showed a difference in average percent green cover, which also supports this lack of significant relationship.

In trials 3 and 4, digital imaging analysis found little to no relationship between number of billbugs and turfgrass cover. One of the fundamental differences in trials 3 and 4 is that they were conducted under ideal growth chamber conditions. A potential explanation for the lack of relationship between number of billbugs and turfgrass cover could be the grasses' ability to recover from injury under ideal growing conditions. In this ideal environment, the threshold for number of billbugs to reduce the turfgrass cover is higher than what is observed in trials 1 and 2. This is consistent with what we have seen in natural environments where turfgrass managers typically noticed billbug injury during periods where the grass is under some other type of stress (temperature, moisture, traffic, spring-green up, dormancy, etc.).

The ELISA results indicted that billbugs placed in containers with treated bermudagrass shoot material fed more than when placed in either zoysiagrass or tall fescue. Billbugs that were placed in containers with treated root material from any grass species did not test positive, indicating that billbugs do not feed on root material. The limited number of positive responses in the ELISA could indicate that digestion of the goat IgG occurred before the gut content was analyzed. Future studies should focus on the effects of time of digestion on the persistence of the goat IgG.

This research continues to build the biology and behavior database for the hunting billbug in turfgrass transition zones. Based on the results of these experiments, an integrated pest management program for hunting billbugs in turfgrass transition zones should focus on

monitoring for the presence and activity of adult hunting billbugs. Monitoring for adults requires the installation of pitfall traps or scouting the turfgrass surface at night with a flash light (Silcox et al. 2013). Hunting billbug adult abundance should be monitored primarily in the spring and fall, when the adults are known to be most abundant. This research shows that adult hunting billbugs feed on the shoots of warm-season turfgrass; therefore insecticide applications should be timed with adult activity to reduce the likelihood of warm-season turfgrass damage.

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Tables

Table 1. Analysis of variance for bermudagrass, zoysiagrass, and tall fescue response from application of 0, 2, 4, or 6 billbugs over 5 photograph dates during 4 studies in 2010, 2012, and 2013 and 3 experiments within each study.

Analysis of Variance				
Source	df	Mean Square	*F*	*P* > *F*
Study	3	113537.65	669.39	< 0.0001
Experiment	2	2331.21	13.74	< 0.0001
Grass	2	19620.32	115.68	< 0.0001
Billbugs	3	1200.55	7.08	0.0001
Photograph Date	4	368.22	2.17	0.0701

Table 2. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs in 2010.

Grass	Adult Hunting Billbugs	Average Percent Cover
Bermudagrass	0	60.21 a
Bermudagrass	2	38.09 d
Bermudagrass	4	45.22 c
Bermudagrass	6	54.23 b
P-Value	<0.0001	

Data were analyzed using Proc GLM and means were separated using Fisher's Protected LSD test. Means followed by the same letter are not different ($P < 0.05$).

Table 3. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2010.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	45.85 b	39,91 a	44.66 a	46.02 b
Photograph Date 2	50.84 b	46.66 a	53.26 a	57.9 a
Photograph Date 3	65.89 a	38.46 a	42.79 a	60.12 a
Photograph Date 4	71.15 a	35.53 a	54.02 a	63.56 a
P-Value	0.0008	0.6116	0.3714	0.0336

Table 4. Average percent cover of zoysiagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs in 2010.

Grass	Adult Hunting Billbugs	Average Percent Cover
Zoysiagrass	0	56.42 a
Zoysiagrass	2	40.99 c
Zoysiagrass	4	48.01 b
Zoysiagrass	6	50.78 b
P-Value	<0.0001	

Data were analyzed using Proc GLM and means were separated using Fisher's Protected LSD test. Means followed by the same letter are not different ($P < 0.05$).

Table 5. Average percent cover of zoysiagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2010.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	50.85 a	40.92 a	51.49 a	46.00 a
Photograph Date 2	47.33 a	42.93 a	49.45 a	47.85 a
Photograph Date 3	55.83 a	29.84 a	44.74 a	47.82 a
Photograph Date 4	61.55 a	40.73 a	42.51 a	49.49 a
P-Value	0.2623	0.2056	0.6931	0.9573

Table 6. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs in 2012.

Grass	Adult Hunting Billbugs	Experiment A	Experiment B	Experiment C
Bermudagrass	0	41.60 a	21.64 a	35.79 a
Bermudagrass	2	32.86 bc	20.82 a	27.97 b
Bermudagrass	4	36.15 b	13.46 b	23.02 b
Bermudagrass	6	30.48 c	10.26 b	23.75 b
P-Value		<0.0001	<0.0001	0.0044

Data were analyzed using Proc GLM and means were separated using Fisher's Protected LSD test. Means followed by the same letter are not different ($P < 0.05$).

Table 7. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2012 for experiment A.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	51.05 a	45.77 a	49.09 a	53.46 a
Photograph Date 2	51.86 a	38.47 ab	46.56 ab	40.08 b
Photograph Date 3	45.97 a	34.06 b	36.88 bc	26.75 c
Photograph Date 4	32.72 a	25.05 c	27.73 dc	18.42 c
Photograph Date 5	26.39 a	20.95 c	20.47 d	13.70 c
P-Value	< 0.0001	< 0.0001	0.0004	< 0.0001

Table 8. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2012 for experiment B.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	22.05 a	20.41 a	19.44 a	18.25 a
Photograph Date 2	20.70 a	19.63 a	8.53 a	8.54 a
Photograph Date 3	20.42 a	19.37 a	6.91 a	5.33 a
Photograph Date 4	23.21 a	20.92 a	22.44 a	6.90 a
Photograph Date 5	21.82 a	23.82 a	10.00 a	12.28 a
P-Value	0.9893	0.9002	0.1053	0.1080

Table 9. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2012 for experiment C.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	26.36 b	16.44 b	15.51 b	24.35 a
Photograph Date 2	30.27 b	19.14 b	15.662 b	20.91 a
Photograph Date 3	53.73 a	46.31 a	44.97 a	37.53 a
Photograph Date 4	27.80 b	29.75 b	15.97 b	22.68 a
Photograph Date 5	40.78 ab	28.21 b	23.04 b	13.27 a
P-Value	0.0101	0.0065	0.0454	0.1464

Table 10. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs in 2012.

Grass	Adult Hunting Billbugs	Experiment A	Experiment B	Experiment C
Tall Fescue	0	25.22	33.55	52.46
Tall Fescue	2	20.41	33.94	44.55
Tall Fescue	4	19.49	33.45	50.13
Tall Fescue	6	20.44	33.98	50.51
P-Value		0.4139	0.9961	0.2583

Data were analyzed using Proc GLM and means were separated using Fisher's Protected LSD test.

Table 11. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2012 for experiment A.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	29.54 a	32.60 a	35.00 a	33.18 a
Photograph Date 2	24.23 a	22.61 ab	23.75 ab	20.43 a
Photograph Date 3	27.81 a	20.35 ab	17.71 ab	20.70 a
Photograph Date 4	22.56 a	14.63 b	11.85 b	15.10 a
Photograph Date 5	21.97 a	11.81 a	9.15 b	12.80 a
P-Value	0.9354	0.0547	0.0544	0.0739

Table 12. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2012 for experiment B.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	37.68 ab	42.64 a	43.44 a	41.82 a
Photograph Date 2	26.99 c	26.96 b	25.23 a	26.13 b
Photograph Date 3	30.30 bc	28.98 b	26.48 a	28.14 b
Photograph Date 4	n/a	n/a	n/a	n/a
Photograph Date 5	39.24 a	37.20 ab	38.64 a	39.83 a
P-Value	0.0158	0.0585	0.0713	0.0042

Table 13. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2012 for experiment C.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	42.69 b	36.63 a	41.79 a	41.96 b
Photograph Date 2	45.36 b	37.81 a	42.37 a	46.24 ab
Photograph Date 3	36.04 b	46.18 a	47.87 a	39.92 b
Photograph Date 4	71.86 a	52.82 a	60.42 a	62.42 a
Photograph Date 5	66.33 a	49.33 a	58.18 a	62.04 a
P-Value	0.0013	0.5452	0.1737	0.0241

Table 14. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs in 2013.

Grass	Adult Hunting Billbugs	Experiment A	Experiment B	Experiment C
Bermudagrass	0	62.96 a	53.62 a	47.12 a
Bermudagrass	2	55.42 a	52.03 a	42.15 b
Bermudagrass	4	61.53 a	53.69 a	48.72 a
Bermudagrass	6	61.94 a	52.00 a	48.4 a
P-Value		0.0863	0.8777	0.0135

Data were analyzed using Proc GLM and means were separated using Fisher's Protected LSD test. Means followed by the same letter are not different ($P < 0.05$).

Table 15. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2013 for experiment A.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	62.30 a	57.74 a	65.27 a	56.92 a
Photograph Date 2	61.58 a	53.89 a	59.88 a	62.66 a
Photograph Date 3	62.79 a	53.30 a	61.46 a	62.19 a
Photograph Date 4	67.10 a	58.43 a	65.28 a	68.09 a
Photograph Date 5	61.01 a	53.73 a	55.77 a	59.86 a
P-Value	0.4256	0.9706	0.1164	0.7941

Table 16. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2013 for experiment B.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	58.25 a	55.76 a	56.25 a	55.34 a
Photograph Date 2	51.45 a	50.69 a	51.57 a	48.73 a
Photograph Date 3	55.92 a	56.06 a	58.27 a	52.02 a
Photograph Date 4	51.81 a	54.64 a	52.54 a	53.85 a
Photograph Date 5	50.67 a	42.98 a	49.85 a	50.02 a
P-Value	0.7651	0.0870	0.6314	0.8840

Table 17. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2013 for experiment C.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	48.06 a	40.97 a	49.00 a	50.28 ab
Photograph Date 2	54.14 a	45.75 a	50.31 a	53.41 a
Photograph Date 3	50.66 a	46.06 a	50.52 a	50.36 ab
Photograph Date 4	43.38 a	41.12 a	47.03 a	45.60 bc
Photograph Date 5	39.37 a	36.86 a	46.76 a	42.34 c
P-Value	0.2519	0.5618	0.3463	0.0054

Table 18. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs in 2013.

Grass	Adult Hunting Billbugs	Experiment A	Experiment B	Experiment C
Tall Fescue	0	82.43	77.64	73.69
Tall Fescue	2	81.84	78.37	69.50
Tall Fescue	4	81.01	78.40	72.01
Tall Fescue	6	77.39	79.26	70.70
P-Value		0.1780	0.8803	0.0602

Data were analyzed using Proc GLM and means were separated using Fisher's Protected LSD test.

Table 19. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2013 for experiment A.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	78.51 a	83.07 ab	80.17 a	74.84 a
Photograph Date 2	87.14 a	87.10 a	81.95 a	80.69 a
Photograph Date 3	83.14 a	82.30 ab	84.37 a	81.59 a
Photograph Date 4	82.46 a	78.46 b	81.85 a	74.90 a
Photograph Date 5	80.90 a	78.25 b	76.74 a	75.29 a
P-Value	0.8279	0.0267	0.7109	0.5311

Table 20. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2013 for experiment B.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	79.47 a	80.86 a	78.95 a	80.17 a
Photograph Date 2	78.31 a	80.01 a	79.52 a	81.32 a
Photograph Date 3	78.99 a	80.58 a	79.29 a	81.06 a
Photograph Date 4	76.31 a	76.28 a	78.19 a	78.12 a
Photograph Date 5	75.10 a	74/09 a	76.04 a	75.62 a
P-Value	0.6358	0.8093	0.8353	0.4226

Table 21. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2013 for experiment C.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	78.07 a	73.26 a	74.14 a	70.26 a
Photograph Date 2	73.02 a	71.23 a	74.95 a	69.42 a
Photograph Date 3	73.41 a	66.95 a	73.05 a	72.68 a
Photograph Date 4	69.47 a	67.48 a	69.67 a	69.17 a
Photograph Date 5	74.49 a	66.95 a	68.23 a	71.98 a
P-Value	0.4444	0.5883	0.0600	0.4177

Table 22. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs in 2013.

Grass	Adult Hunting Billbugs	Experiment A	Experiment B
Bermudagrass	0	36.32	33.89
Bermudagrass	2	36.59	30.65
Bermudagrass	4	33.66	33.52
Bermudagrass	6	38.29	28.33
P-Value		0.4404	0.2562

Data were analyzed using Proc GLM and means were separated using Fisher's Protected LSD test.

Table 23. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2013 for experiment A.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	45.21 a	39.88 a	47.53 a	48.58 a
Photograph Date 2	39.29 a	41.90 a	36.53 ab	39.00 ab
Photograph Date 3	36.83 a	36.99 a	30.07 b	36.94 ab
Photograph Date 4	29.01 a	32.12 a	27.71 b	32.84 b
Photograph Date 5	31.25 a	32.05 a	26.45 b	34.10 b
P-Value	0.0828	0.6289	0.0106	0.1233

Table 24. Average percent cover of bermudagrass using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2013 for experiment B.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	40.52 a	37.19 a	42.97 a	30.86 a
Photograph Date 2	36.37 a	31.25 a	36.14 a	27.84 a
Photograph Date 3	31.34 a	26.80 a	27.50 a	25.01 a
Photograph Date 4	29.15 a	27.46 a	27.59 a	27.02 a
Photograph Date 5	32.08 a	30.54 a	33.40 a	30.90 a
P-Value	0.3745	0.7875	0.3031	0.6200

Table 25. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs in 2013.

Grass	Adult Hunting Billbugs	Experiment A	Experiment B
Tall Fescue	0	25.92 b	30.48 a
Tall Fescue	2	31.19 b	25.85 c
Tall Fescue	4	31.47 b	25.89 bc
Tall Fescue	6	41.03 a	29.49 ab
P-Value		<0.0001	0.0194

Data were analyzed using proc GLM and means were separated using Fisher's Protected LSD test. Means followed by the same letter are not different ($P < 0.05$).

Table 26. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2013 for experiment A.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	25.21 a	27.87 a	28.84 a	36.45 a
Photograph Date 2	26.36 a	30.51 a	32.59 a	39.38 a
Photograph Date 3	25.77 a	29.47 a	30.78 a	40.36 a
Photograph Date 4	24.36 a	30.98 a	30.45 a	40.70 a
Photograph Date 5	27.90 a	37.14 a	34.71 a	48.24 a
P-Value	0.9218	0.8837	0.7167	0.6664

Table 27. Average percent cover of tall fescue using digital image analysis after application of 0, 2, 4, or 6 adult hunting billbugs over 4 photograph dates in 2013 for experiment B.

	Average Percent Green Cover			
	0 Billbug	2 Billbug	4 Billbug	6 Billbug
Photograph Date 1	24.04 b	21.96 b	23.80 a	26.84 a
Photograph Date 2	27.27 b	25.67 ab	25.21 a	30.35 a
Photograph Date 3	26.53 b	24.47 ab	23.53 a	26.90 a
Photograph Date 4	32.60 ab	26.93 ab	26.88 a	30.80 a
Photograph Date 5	41.98 a	30.21 a	30.04 a	32.56 a
P-Value	0.0308	0.2837	0.1019	0.5800

Table 28. ELISA testing for purified goat IgG applied to either the shoots or roots of bermudagrass, zoysiagrass or tall fescue at 50 µl goat IgG in 50 ml phosphate buffered saline.

Grass	Treated Part	*n*	Number of Positive Reactions	ELISA Optical Density Range		Critical Value $\bar{x} \pm 4$ SD
				Treated Beetle	Untreated Beetle	
Bermudagrass	Shoots	12	6	0.155-0.265	0.155-0.178	0.186
Bermudagrass	Roots	12	0	1.067-2.981	0.529-2.841	3.09
Zoysiagrass	Shoots	12	1	0.240-0.394	0.223-0.302	0.391
Zoysiagrass	Roots	12	0	0.187-0.220	0.146-0.220	0.225
Tall Fescue	Shoots	12	0	0.108-0.266	0.166-0.203	0.326
Tall Fescue	Roots	12	0	0.089-0.261	0.164-0.220	0.279

Conclusions

The results of these studies provide a better understanding of hunting billbug biology and behavior in warm-season turfgrass. This information has allowed us to develop a management plan that focuses on season-long monitoring of adult hunting billbugs. Monitoring for adult hunting billbug includes installing pitfall traps and checking the traps weekly for the presence of adult billbugs. A second option is to scan the turfgrass surface, weekly, 30 min after sunset with a flashlight for the presence of adult billbugs. This will inform turfgrass managers when adults become active in the spring. Based on this research we know that once the adults are active in the spring, the females begin to oviposit viable eggs. Tracking rain events throughout the spring and summer will inform managers of larval survival likelihood. Continuing to monitor adult populations all year will allow managers to determine when the adult billbug populations begin to increase. If visible damage is associated with adult hunting billbug feeding on shoot material, a treatment with a pyrethroid or neonicotinoid product when adults have reached peak abundance will maximize effectiveness of application and reduce environmental inputs.