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Billbug (Sphenophorus spp.) chemical ecology and seasonal biology in Indiana turfgrass

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BILLBUG (*SPHENOPHORUS* spp.) CHEMICAL ECOLOGY AND SEASONAL BIOLOGY IN INDIANA TURFGRASS

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This thesis is dedicated to the late Dr. Ernest Daniels.

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ABSTRACT

Duffy, Alexandra G. M.S., Purdue University, December 2016. Billbug (*Sphenophorus* spp.) Chemical Ecology and Seasonal Biology in Indiana Turfgrass. Major Professor: Douglas S. Richmond.

Billbugs (Coleoptera: Curculionoidea: *Sphenophorus*) are serious pests of managed turfgrass across North America. Damage symptoms are most visible during stressful periods of the growing season and are commonly confused with disease, drought, or nutrient deficiency. Billbugs are frequently a perennial problem and when misdiagnosed, damage often results in seriously degraded stands of turfgrass that are easily encroached by weeds. Presently, management of billbugs relies heavily on chemical insecticides. Even then, the nationwide assemblage of multiple sympatric billbug species and the cryptic nature of the damaging larval stage makes management of these insects challenging. A better understanding of billbug biology and behavior could improve the efficacy of insecticide inputs and provide a basis for the development of alternative, non-pesticide management techniques that are aligned with integrated pest management (IPM). This thesis focused on characterizing the billbug species composition in Indiana and clarifying the seasonal phenology of one particularly problematic species, *S. venatus*. To accomplish this, I examined the utility of molecular techniques to identify the otherwise cryptic larval stage of several sympatric, turf-inhabiting billbug species. I also explored the potential for *S. venatus* to use two forms of

chemoreception: recognition of volatile organic compounds and cuticular wax components.

Weekly monitoring of pitfall traps revealed four sympatric billbug species in Indiana: *S. venatus*, *S. parvulus*, *S. minimus*, and *S. inaequalis*. Further investigation on the seasonal biology of *S. venatus* revealed two overlapping cohorts in Indiana and molecular confirmation of overwintering *S. venatus* larvae through examination of three genetic loci (CO1, 18S, and ITS2). In y-tube olfactometer bioassays, *S. venatus* males were attracted to the combination of conspecifics and host-plants as well as host-plants alone. *S. venatus* females were attracted to the combination of male conspecifics and host plant material and male conspecifics alone. These findings suggest *S. venatus* males are predominantly influenced by host-plant volatiles while females likely respond to a male-produced volatile pheromone. Coupled gas chromatography-mass spectrometry (GC-MS) analysis of *S. venatus* and *S. parvulus* whole-body cuticular extracts revealed a series of aliphatic hydrocarbons with qualitative and quantitative interspecific differences, as well as intraspecific quantitative differences between males and females. These differences in cuticular hydrocarbon profiles could serve as critical mate-recognition cues among sympatric *Sphenophorus* species, a hypothesis that remains to be tested.

By clarifying the seasonal phenology of *S. venatus*, results provide a foundation for improved insecticide selection and application timing in the Midwestern U.S. for this pest. Furthermore, findings demonstrate that a DNA-based larval identification tool could be useful for clarifying the seasonal phenology of sympatric billbug species with morphologically indistinguishable larval stages. Findings also support the idea that

volatile and contact semiochemicals could potentially be used for the development of improved billbug monitoring techniques and sustainable mating disruption strategies.

LITERATURE REVIEW

1.1 Genus *Sphenophorus*

Weevils (Coleoptera: Curculionoidea) are a superfamily of beetles that utilize most plant structures of virtually all described plant taxa, making them both ecologically significant and one of the most economically important groups of agricultural insect pests. The genus *Sphenophorus* Schönherr is historically associated with a diverse assortment of sedges and grasses. It consists of 71 described species, commonly referred to as billbugs, with 64 being indigenous to North America (Vaurie 1951).

Billbug adults are hard bodied, generally grey, black, or reddish-brown, with chewing mouthparts distally located on a characteristic long snout, or rostrum, that is at least half the length of the pronotum (Vittum et al. 1999, Young 2002). *Sphenophorus* spp. adults are distinguished from other weevil genera by several morphological characters, including the bulbous shape of the antennal club, the relative separation of the coxae, the shape of the mesoepimeron, metaepimeron, and intercoxal processes, tibial projection shape, and ventral setae of the third tarsal segment (Vaurie 1951). The antennae of *Sphenophorus* spp. are attached near the base of the snout, which distinguishes them from the only other genus of economically important weevils known to be injurious to managed turfgrass, *Listronotus* spp., where the antennae are attached near the distal end of the snout (Vittum et al. 1999). The markings and indentations on

the pronotum, elytra, abdominal sclerites, and profemur are useful for identifying adult billbug species (Vaurie 1951, Johnson-Cicalese 1990, Shetlar 2011). Although for most species, adults rarely cause economic damage to agriculturally important grasses, the preponderance of studies describing billbug seasonal ecology have focused on adults because they can be readily identified to species based on external morphological characters.

There are also several key characteristics useful for distinguishing the pupae of billbug species; primarily setae, the length of the rostrum, and the width of the pronotum (Satterthwait 1931). Billbug eggs are oblong, creamy white, glossy, and typically 1-2 mm in length (Vittum et al. 1999) but are of little utility for identifying billbug species. Anderson (1948) provided a generic key to larvae of the subfamily Calendrinae (now Dryophthorinae), which included the genus *Calendra* (now *Sphenophorus*), but did not progress to the species level for most. In general, billbug larvae are white, legless, grub-like larvae with a brown head capsule (Anderson 1948, Vittum et al. 1999). Because there are currently no published external morphological characteristics to distinguish billbug larvae, our understanding of billbug seasonal phenology is limited.

1.2 *Sphenophorus* spp. in managed turfgrass

Billbugs were first recognized as a serious pest of turfgrass in the 1960s after an outbreak of the bluegrass billbug, *Sphenophorus parvulus* Gyllenhaal, across several states (Tashiro and Personius 1970). Now, at least ten species within *Sphenophorus* are known to infest managed turfgrass: the bluegrass billbug *S. parvulus* Gyllenhaal, the lesser billbug *S. minimus* Hart, the uneven billbug, *S. inaequalis* Say, the hunting billbug

S. venatus Say, the Phoenician billbug *S. phoeniciensis* Chittenden, the Rocky Mountain billbug *S. cicatristriatus* Fahraeus, the Southern corn billbug *S. callosus* Olivier, *S. coesifrons* Gyllenhaal, *S. apicalis* LeConte, *S. rectus* Say, and *S. cariosus* Olivier (Vaurie 1951, Morrill and Suber 1976, Johnson-Cicalese et al. 1990, Dupuy and Ramirez 2016). An overview of the distributions of *Sphenophorus* species associated with turfgrass across 11 regions of the U.S. revealed the possibility for many sympatric *Sphenophorus* species (Johnson-Cicalese 1990). A more recent review by Dupuy and Ramirez (2016) outlined the distribution of eleven *Sphenophorus* species and their common turfgrass hosts. They cited four species that dominated different regions of the United States: *S. venatus* in the Southeast, *S. parvulus* in the Northeast and Midwest, *S. phoeniciensis* in the Southwest, and *S. cicatristriatus* in the Rocky Mountain region. Studies in South Carolina (Chong 2015), Virginia (Kuhn et al. 2013), North Carolina (Doskocil and Brandenburg 2012), and Florida (Huang and Buss 2009) have also indicated billbug species assemblages in both warm- and cool-season turfgrasses. Four sympatric species occur in the east central U.S.: *S. parvulus*, *S. venatus*, S. *inaequalis*, and *S. minimus* (Johnson-Cicalese et al. 1990). Of these species, *S. parvulus* and *S. venatus* are the most important pests. However, the occurrence of several sympatric billbug species in many regions of the U.S and the lack of morphological characters useful for identifying billbug larvae complicates efforts to clarify the seasonal phenology of billbugs associated with turfgrass systems.

1.3 The bluegrass billbug (*S. parvulus*)

Bluegrass billbugs are typically 5 to 8 mm long with dense, uniform punctures on the pronotum, and sometimes a narrow, slightly raised, non-punctuated median line. The elytra are covered in alternating rows of small and large punctures (Vaurie 1951, Vittum et al. 1999, Richmond 2016). Bluegrass billbugs are significant pests of cool-season grasses and are widely distributed across the continental United States. They can generally be found wherever Kentucky bluegrass (*Poa pratensis*) is grown, but they are known to infest other cool-season turfgrass species such as perennial ryegrass (*Lolium perenne*), tall fescue (*Festuca arundinacea*), and fine fescue (*Festuca* sp.). They are also frequently found in low abundance within species assemblages occurring on warm-season turfgrasses, such as Bermudagrass (*Cynodon* spp.) and zoysiagrass (*Zoysia* spp.) (Vittum et al. 1999, Dupuy and Ramirez 2016). The bluegrass billbug overwinters in the adult stage in the thatch, soil, and plant debris. Adults become active as soil surface temperatures warm in April or May and typically have one generation a year, although a partial second generation is possible (Vittum et al. 1999). Bluegrass billbug adults feed by chewing on turfgrass stems, but damage from adult feeding has not been documented in turfgrass. Females oviposit into turfgrass stems and early instar larvae feed within the plant. As larvae develop, they move into the crown at the base of the stem where they can be found just below the soil surface and in root zone from mid-June through July. This feeding pattern causes the plants to break off easily from the soil surface, leaving behind a diagnostic sawdust-like frass (Vittum et al. 1999, Richmond 2016).

1.4 The hunting billbug (*S. venatus*)

Hunting billbug adults are slightly larger than bluegrass billbugs, ranging from 8-11 mm in length. Additionally, the hunting billbug is differentiated by its coarsely, non-uniform punctuated pronotum with a definitive smooth, non-punctuated Y-shaped median area surrounded by parenthesis-like curved side markings, or vittae (Vaurie 1951, Johnson-Cicalese 1990). Vaurie (1951) recognized five subspecies of *S. venatus*: *S.v. venatus, S.v. vestita, S.v.glyceriae, S.v. confluens,* and *S.v. reticulaticollis.* The common name "hunting billbug" is typically associated with the subspecies *S. venatus vestitus*, but because this subspecies complex was described predominantly based on geographic distribution and not basic diagnostic external or reproductive morphological differences, I refer to the hunting billbug simply by the species name, *S. venatus*.

The hunting billbug is primarily a pest of warm-season grasses in the southeastern United States such as Florida (Huang and Buss 2009), Arkansas (Young 2002), South Carolina (Chong 2015), and North Carolina (Doskocil and Brandenburg 2012, Reynolds et al. 2015). However, it has also been documented further north into Virginia (Kuhn et al. 2013) and New Jersey (Johnson-Cicalese et al. 1990), west into Indiana (Richmond 2016), Kansas (Brussel and Clark 1968), Idaho, Utah (Dupuy and Ramirez 2016), southern California (Vittum et al. 1999), Mexico (León-García et al. 2012, Ordaz-González 2014), Hawaii (Vittum et al. 1999) and Japan (Yoshia and Nabeshima 1981). The hunting billbug is most commonly found on Bermudagrass (*Cynodon* spp.) and zoysiagrass (*Zoysia* spp.). However, it is a pest of multiple warm- and cool-season turfgrass species, such as St. Augustine grass (*Stenotaphrum secundatum*), centipedegrass (*Eremochloa ophiuroides*), tall fescue, perennial ryegrass, and Kentucky bluegrass,

making it an important pest within the turfgrass climatic transition zone, where both warm- (C4) and cool-season (C3) grasses are grown (Johnson-Cicalese and Funk 1990, Vittum et al. 1999, Huang and Buss 2009, Doskocil and Brandenburg 2012, Dupuy and Ramirez 2016).

It has been suggested that the hunting billbug overwinters as both adults and larvae in warm- and cool-season turfgrass in its southern-most range in Florida (Young 2002), northern parts of its range in New Jersey (Johnson-Cicalese et al. 1990), and within the transition zone in North Carolina (Doskocil and Brandenburg 2012, Reynolds et al. 2015). However, *Sphenophorus* larvae are not morphologically distinguishable from other species, so these claims are substantiated based solely on the observation of a large abundance of hunting billbug adults within a given sampling area. Two overlapping generations per year have been reported in North Carolina (Doskocil and Brandenburg 2012) and as many as six overlapping generations per year have been observed in Florida (Huang and Buss 2009). It is currently assumed that the oviposition behavior and larval development of hunting billbug is similar to that of the bluegrass billbug, although hunting billbug adults are predominantly nocturnal (Huang 2008). Unlike the bluegrass billbug, where visible turfgrass damage is associated with the larvae (Vittum et al. 1999), damage as a result of large hunting billbug infestations has also been associated with adult activity (Huang and Buss 2013). In North Carolina, hunting billbug adults significantly reduced the greenness and height of both warm-season and cool-season turfgrass (Doskocil & Brandenburg 2012, Reynolds et al. 2013).

1.5 The lesser (*S. minimus*) and uneven (*S. inaequalis*) billbugs

The lesser (*S. minimus*) and uneven (*S. inaequalis*) billbugs are not as extensively studied as the bluegrass and hunting billbugs. Adult *S. inaequalis* billbugs are proportionally broader than other billbug species, but about the same length as the bluegrass billbug (7-8 mm). The pronotum contains an elongated diamond-shaped, non-punctate medial area and is covered with unevenly spaced punctures that are not uniformly sized. The lesser billbug is generally lighter in color and smaller (6-7 mm in length) with the pronotum covered in larger, non-uniform, sparse punctures (Vaurie 1951, Johnson-Cicalese et al. 1990, Richmond 2016). Neither species is typically very abundant and they are most often found in mixed populations on both warm- and cool-season turfgrasses with the bluegrass billbug, hunting billbug, and sometimes several other species (Johnson-Cicalese et al. 1990, Dupuy and Ramirez 2016). Two notable exceptions to this general pattern have been reported, where *S. inaequalis* was the most abundant billbug species or found in equal abundance with *S. parvulus*, *S. venatus,* and *S. minimus*, on cool-season grasses in New Jersey (Johnson-Cicalese et al. 1990) and Bermudagrass in Florida (Huang and Buss 2013a). These two species, similar to the bluegrass billbug, are thought to be univoltine throughout their range (Richmond 2016, Huang and Buss 2013a, Johnson-Cicalese et al. 1990).

1.6 Billbug damage, diagnosis, and monitoring

Billbug damage is arguably the most widely misdiagnosed insect-related turfgrass disorder in North America, frequently resulting in unnecessary and ineffective herbicide and fungicide inputs. Billbug damage symptoms are similar for all billbug species, first

appearing as small spots (5-8 cm in diameter) of brown, dying turfgrass, which sometimes coalesce to form large, irregular patches. These damage symptoms are often confused with dollar spot disease, spring dead spot disease, drought, nutrient deficiency, delayed spring green-up, or summer dormancy (Vittum et al. 1999). As a result, billbugs can become a perennial problem and accumulation of this damage may result in seriously degraded turfgrass stands that are easily encroached by weeds (Richmond et al. 2000). Adult billbugs are stem-boring and females will oviposit into holes chewed in the plant stems. For the hunting billbug, *S. venatus* Say, adult feeding and oviposition behavior contributes to damage in warm-season grasses (Doskocil and Brandenburg 2012, Reynolds et al. 2015) as damage symptoms will often be reported during peak adult activity periods (Huang and Buss 2009). Larval damage has also been well-documented. In cool-season turfgrasses, larvae are the only known damaging life stage, with smaller larvae feeding inside plant stems and larger larvae feeding on the crowns, roots, rhizomes, and stolons (Vittum et al. 1999).

Larval feeding leaves behind a diagnostic sawdust-like frass and causes stems to easily break away from the plant crowns at the soil surface (Vittum et al. 1999, Richmond 2016). Larvae can be detected directly using a golf course cup cutter, knife, or shovel to sample the soil to a depth of ~9 cm, breaking apart the soil, and examining the crowns and roots for larvae. Billbug adults are not strong fliers (Young 2002), but rather walk as their main means of dispersal. Bluegrass billbugs are often observed on driveways, sidewalks, cart paths and curbs as they disperse in the spring and late summer. Scouting these areas, or pitfall trapping, can be used to monitor adult activity (Richmond 2016).

1.7 Billbug management

Billbug management relies on a combination of cultural, biological and chemical tools. Billbug damage is most evident in stressed turf, therefore, increased mowing heights, providing adequate fertilization, optimal irrigation, thatch management and cultivation can promote healthy, vigorous turf that is capable of withstanding or quickly recovering from moderate billbug infestations (Vittum et al. 1999, Dupuy and Ramirez 2016, Richmond 2016). Resistant turfgrass varieties are less likely to suffer damage and are quicker to recover. Endophyte-enhanced (E+) turfgrasses harbor symbiotic fungi (*Epichloe* spp.) that provide resistance to billbug adults and larvae through feeding deterrence and delayed development, as well as improved tolerance to other environmental stresses (Johnson-Cicalese and White 1990, Vittum et al. 1999, Richmond 2016). A stand of turfgrass composed of 40% E+, plants, which can often be accomplished by overseeding E+ varieties into pre-existing turfgrass stands, is recommended (Richmond et al. 2000). In addition, several varieties of Kentucky bluegrass exhibit resistance to bluegrass billbugs due to aggressive growth or finer texture and narrower stems that are not preferred for oviposition (Vittum et al. 1999, Fry and Cloyd 2011, Dupuy and Ramirez 2016). Some varieties of Bermudagrass and zoysiagrass provide resistance to hunting billbugs (Reinert et al. 2011, Huang and Buss 2013b).

Commercially available biological control agents are largely limited to two entomopathogenic nematodes, *Heterohabditis bacteriophora* and *Steinernema carpocapsae* (Dupuy and Ramirez 2016, Richmond 2016). Entomopathogenic nematodes are known to suppress both white grubs and billbugs (Niemczyk and Shetlar 2000). *H.*

bacteriophora, a soil-active “cruiser-type” nematode is effective against billbug larvae once they have entered the root zone, whereas *S. carpocapsae*, a surface-active “ambusher-type” nematode, is more effective against adult billbugs (Table 1.1) (Richmond 2016).

Management of billbugs still relies heavily on chemical intervention. Three insecticide-based management strategies, targeting different developmental life stages have been widely adopted: 1) preventative application of contact insecticides, such as pyrethroids, carbamates, and organophosphates, targeting overwintered adults prior to oviposition in the early spring, 2) preventative application of plant systemically active insecticides, such as neonicotinoids and anthranilic diamides, to control adults on the surface and early instar larvae inside the stems and 3) curative application of soil insecticides, such as neonicotinoids, carbamates and organophosphates, targeting late instar larvae in the soil after damage has been diagnosed (Richmond 2016, Shetlar and Andon 2012). The implementation of these strategies is largely dependent upon accurately timed insecticide applications, which may vary regionally with billbug species composition and seasonal phenology (Reynolds and Brandenburg 2015).

1.8 Molecular life stage associations in Coleoptera

The integration of DNA to associate different developmental stages can be especially useful in systems where larvae cannot be readily identified based on morphological characters or rearing larvae to the adult stage is difficult. Molecular methods have been utilized to associate the adult and larval stages of morphologically cryptic beetles (Miller et al. 2005) and assemblages of multiple sympatric beetle species

(Ahrens et al. 2007, Doskocil et al. 2008). Doskocil et al. (2008) examined the assemblage and molecular life-stage association of white grubs (Coleoptera: Scarabaeidae), another insect species complex that is economically harmful to turfgrass (Doskocil et al. 2008). DNA from mitochondrial genes, most often cytochrome oxidase *c* subunit 1 (COI), is the most common method for life-stage associations. COI is particularly useful due to the rapidly evolving nature of the gene, which theoretically minimizes genetic variation within species, and allows closely-related species to be well resolved (Ahrens et al. 2007). However, the use of a single gene region is often criticized and the use of several genetic regions for associating life stages is preferred. Li et al. (2007) used 18S ribosomal DNA to diagnose two species of *Smicronyx* (Coleoptera: Curculionidae). The second internal transcribed spacer region (ITS2) successfully distinguishes among closely-related mosquito species (Marrelli et al. 2006), but it's effectiveness for associating beetle life stages has not been investigated. If DNA-based techniques could be employed to clarify the larval population dynamics of sympatric, turf-inhabiting billbug species, they could promote the development of more efficient monitoring and management programs for the billbug species complexes occurring in different regions of North America.

1.9 Volatile chemical communication in Curculionoidea/*Sphenophorus*

The use of long-range, volatile chemical signals, such as host-plant volatile organic compounds (VOCs) or pheromones, is crucial for intra- and inter-specific communication across many orders of insects (Thornhill & Alcock 1983). Beetles (Insecta: Coleoptera) are highly diverse in the chemical structure and biological

significance of these volatile signals. The majority of weevils (Coleoptera: Curculionoidea) that use volatile, chemically-mediated attraction, use male-produced aggregation (Landolt 1997). The four-part male-produced aggregation pheromone blend of the boll weevil, *Anthonomus grandis* Boheman, was the first weevil pheromone identified (Francke and Dettner 2005).

Within Dryophthoridae (Coleoptera: Curculionoidea), volatile male-produced aggregation pheromones, which often mediate interspecific attraction (Francke and Dettner 2005), have been identified for *Sitophilus* Schönherr (Schmuff et al. 1984), *Rhynchophorus* Herbst (Giblin-Davis et al. 1996), *Scyphophorus* Schönherr (Ruiz-Montiel et al. 2003), and *Sphenophorus* Schönherr (Zarbin et al. 2003, Illescas-Riquelme et al. 2016). *Sphenophorus levis* Vaurie and *S. incurrens* Gyllenhaal, the only two species within the genus *Sphenophorus* whose chemoreception has been previously studied, produce a male-specific chiral compound, 2-methyl-4-octanol, that is attractive to both sexes. However, unlike many closely-related species, (S)-2-methyl-4-octanol was the sole chemical component for these two species (Zarbin et al. 2003, Illescas-Riquelme et al. 2016), and it was not part of a multi-component blend. 2- methyl-4-octanol is also an important pheromone component for *Metamasius hemipterus* Linnaeus, where it is accompanied by 2-methyl-4-heptanol, 4-methyl-5-nonanol, and the corresponding ketones, 5-nonanol, and 3-hydroxy-4-methyl-5- nonanone. 2-methyl-4-octanol is also present in ratio-specific pheromone blends for the Australian population of *Rhabdoscelus obscurus* Boisduval (Giblin-Davis et al. 2000), and *Scyphophorus acupunctatus* Gyllenhaal (Ruiz-Montiel et al. 2008).

Many weevils, such as the boll weevil *A. grandis* (Dickens 1984), the cranberry weevil *Anthonomus musculus* Say (Szendrei et al. 2009), and the annual bluegrass weevil, *Listronotus maculicollis* Kirby, respond to host-plant VOCs. *L. maculicollis* females exhibit both behavioral and electroantennographic (EAG) responses to *Poa annua* host-plant volatiles, while males only displayed EAG responses. Although both sexes possess receptor neurons for volatiles released by *P. annua,* their behavioral responses differed. Behavioral differences between sexes in response to host-plant volatiles are commonly observed among insects (Szendrei and Rodriguez-Saona 2010). Host-plant volatiles frequently have synergistic effects with male-produced aggregation pheromones in weevils (Rochat et al. 2000, Reddy and Guerrero 2004), with attractiveness increasing after males feed on host-plant material (Landolt 1997). Illescas-Riquelme et al. (2016) observed this phenomenon in *Sphenophorus incurrens,* with the pheromone lure + sugarcane host-plant material trapping the most weevils in the field. Although the role of semiochemicals in mediating host- and mate-finding behavior has been examined in several insect pests of turfgrass (Alm et al. 1999, Alm et al. 2006, Potter and Haynes 1993, Robins et al. 2009), chemoreception in billbugs associated with turfgrass has not been previously examined.

1.10 Contact pheromones in Curculionoidea

Close-range semiochemicals are typically non-volatile, contact-perceived stimuli composed of long-chain hydrocarbons within the epicuticular wax layer (Thornhill & Alcock 1983). Insect cuticular hydrocarbons are long-chain hydrocarbons; typically being alkanes, alkenes, and branched alkanes. Aside from protecting the insect from

desiccation, one of the major evolved functions of cuticular hydrocarbons is serving as species- and sex-recognition signals between two or more individuals (Howard and Blomquist 2005).

Hydrocarbon sex pheromones are known from several insect orders, including Coleoptera (Ginzel and Hanks 2003, Ginzel 2010, Hughes et al. 2015). For Curculionoidea, cuticular hydrocarbons serve as contact pheromones for multiple species, including *Diaprepes abbreviates* Linnaeus (Lapointe et al. 2004), *Cylindrocopturus adspersus* LeConte (Pomonis and Hakk 1984), *Aegorhinus supercilious* Guerin (Mutis et al. 2009), and one aquatic weevil *Oryzophagus oryzae* Costa Lima (Martins et al. 2013). The use of these compounds in mating behavior has been confirmed in bioassays with *A. supercilious* (Mutis et al. 2009) and *O. oryzae* (Martins et al. 2013). Antennal contact prior to mounting, and continued tapping or stroking of females with antennae and tarsi once mounted, are mating behaviors that are common in curculionids and other insects that rely on contact chemical signals (Ginzel et al. 2006). Cuticular extractions have also revealed that although males and females frequently share a majority of the same compounds, the relative concentrations may differ (Mutis et al. 2009). The mating sequence prior to copulation, cuticular hydrocarbons, and their potential role as contact semiochemicals has never been reported for *Sphenophorus*.

1.11 Thesis Objectives

Management of billbugs heavily relies on chemical insecticide inputs. However, understanding of regional variation in billbug species composition and seasonal phenology, in particular that of the morphologically undescribed billbug larvae, remains

largely incomplete. In addition, the potential for behavior-manipulating semiochemicals to act as insecticide alternatives or for improving current monitoring techniques, has not been explored. An understanding of basic billbug biology and behavior is necessary to facilitate the development of more sustainable billbug pest management strategies. In working toward this goal, the objectives of this project were to 1) describe the species composition of billbugs infesting turfgrass and clarify *S. venatus* seasonal phenology in Indiana (Chapter 2), 2) utilize DNA-based techniques to identify the larvae of sympatric, turf-inhabiting billbug species (Chapter 2), and 3) test the hypothesis that two forms of chemoreception, recognition of volatile organic compounds and cuticular wax components, mediate billbug behavior (Chapter 3).

Table 1.1 Active ingredients of insecticide products recommended for targeting different life stages of billbugs in turfgrass.

Insecticide (trade names)		**Target life stage**		
		Adult	Larvae in stems	Larvae in soil
SYNTHETIC INSECTICIDES				
Diamide	Chlorantraniliprole (Acelepryn/Syngenta; others)	X	X	
Diamide	Cyantraniliprole (Ference/Syngenta)	X	X	
Neonicotinoid	Clothianidin (Arena/Nufarm; others)	X	X	X
Neonicotinoid	Imidacloprid (Merit/Bayer; others)	X	X	X
Neonicotinoid	Thiamethoxam (Meridian/Syngenta)	X	X	X
Neonicotinoid	Dinotefuran (Zylam/PBI-Gordon)	X	X	
Pyrethroid	Beta-cyfluthrin (Tempo/Bayer)	X		
Pyrethroid	Bifenthrin (Talstar/FMC)	X		
Pyrethroid	Deltamethrin (DeltaGard/Bayer; others)	X		
Pyrethroid	Lambda-cyhalothrin (Scimitar/Syngenta; others)	X		
Pyrethroid	Zeta-cypermethrin (Talstar Xtra/FMC)	X		
Carbamate	Carbaryl (Sevin/Bayer)	X		X
Organophosphate	Chlopyrifos (Dursban/Dow)	X		
Organophosphate	Trichlorfon (Dylox/Bayer)	X		X
BIOLOGICAL INSECTICIDES				
Parasitic Nematode	*Heterorhabditis bacteriphora* (Nemasys G, NemaSeek)			X
Parasitic Nematode	*Steinernema carpocapsae* (Millenium/BASF; others)	X		

SPHENOPHORUS SPECIES COMPOSITION, SEASONAL BIOLOGY, AND DNA-BASED LIFE STAGE ASSOCIATION IN INDIANA TURFGRASS

2.1 Abstract

At least eleven species of billbugs (*Sphenophorus* spp.) are pests of managed turfgrass in North America and the regional variation in species composition and seasonal phenology could have important implications for management. Pitfall trapping at four different locations in Indiana revealed four sympatric species of billbugs: *S. venatus*, *S. parvulus*, *S. minimus*, and *S. inaequalis*, with *S. venatus* (hunting billbug) being the most abundant species on warm-season turfgrasses and *S. parvulus* most abundant on cool-season turfgrasses. Investigation of *S. venatus* seasonal biology in Indiana revealed two overlapping cohorts and molecular confirmation of overwintering *S. venatus* larvae based on three different genetic loci (CO1, 18S and ITS2). Each locus provided varying degrees of utility for differentiating the four billbug species examined, with maximum-likelihood analyses of concatenated sequences, as well as CO1 by itself, providing support for the identity of overwintered larvae as *S. venatus* and monophyletic clades of *S. venatus* and *S. minimus*. Maximum-likelihood trees constructed using only 18S or ITS2 sequences were less informative. Results provided the first direct evidence that *S. venatus* larvae are capable of overwintering in Indiana above 40°N latitude, which may be useful for the development of monitoring and management strategies based on the seasonal phenology of this insect. Findings also clarify the utility of CO1, 18S and ITS2 for studies aimed at

describing billbug larval population dynamics and seasonal phenology in regions where several sympatric billbug species are present.

2.2 Introduction

Billbugs (Coleoptera: Dryophthoridae) were first recognized as a serious pest of turfgrass in the 1960s after an outbreak of the bluegrass billbug, *Sphenophorus parvulus* Gyllenhaal, across several states (Tashiro and Personius 1970). Billbug damage first appear as small spots (5-8 cm in diameter) of brown, dying turfgrass, which coalesce to form large, irregular patches (Vittum et al. 1999). Billbug damage is arguably the most widely misdiagnosed insect-related turfgrass disorder in North America, often being confused for drought, soil compaction, or disease (Vittum et al. 1999). As a result, billbugs can become a perennial problem and accumulation of this damage may result in seriously degraded turfgrass stands that are easily encroached by weeds (Richmond et al. 2000).

Adult billbugs are stem-feeding beetles that chew notches in the grass tiller and then oviposit within the tiller. For the hunting billbug, *S. venatus* Say, this adult feeding and oviposition behavior contributes to damage in warm-season grasses (Doskocil and Brandenburg 2012), but larval damage has also been well-documented. *S. parvulus* Gyllenhaal is the most common pest of cool-season turfgrasses and larvae are the only known damaging life stage. Smaller *S. parvulus* larvae hollow out the stems, leaving a diagnostic sawdust-like frass and stems that are easily broken from the plant crowns at the soil surface. Larger larvae reside in the soil and root zone, feeding on the crowns, roots, rhizomes, and stolons (Vittum et al. 1999).

Successful management of billbugs relies heavily on chemical intervention and three insecticide-based management strategies, targeting different developmental stages, have been widely adopted: 1) preventative application of contact insecticides targeting overwintering adults prior to spring oviposition 2) preventive application of plant systemic insecticides to control adults and early instar larvae inside the stems and 3) curative application of soil insecticides targeting late instar larvae in the soil after damage is visible (Richmond 2016, Shetlar and Andon 2012). Successful management utilizing these strategies is largely dependent upon accurately timed insecticide applications, which may vary regionally with species composition and seasonal phenology.

Sixty-four species of *Sphenophorus* Schönherr are native to North America (Niemczyk and Shetlar 2000), with at least ten species recognized as pests of managed turfgrass (Held and Potter 2012). Historically, management regimes have been based on the biology and ecology of the two most widely distributed pest species, the bluegrass billbug *S. parvulus* in cool-season (C3) grasses, and the hunting billbug *S. venatus* in warm-season (C4) grasses. However, billbug species composition varies regionally, resulting in a nation-wide collage of billbug species assemblages (Dupuy and Ramirez 2015, Johnson-Cicalese 1990). In recent decades, regional variation in adult species composition and seasonal phenology has been documented in Arkansas (Young 2002), Florida (Huang and Buss 2009), New Jersey (Johnson-Cicalese et al. 1990), North Carolina (Doskocil and Brandenburg 2012), South Carolina (Chong 2015), and Virginia (Kuhn et al. 2013). Despite their potential to damage turf, larval populations have been more difficult to characterize, largely due to the co-occurrence of multiple species and the inability to morphologically identify the larvae (Doskocil 2010).

Vaurie's (1951) revision of *Sphenophorus* in United States and Mexico remains the most comprehensive taxonomic reference for the genus to date. Later, Johnson-Cicalese et al. (1990) constructed an illustrated key to eight *Sphenophorus* turf pests in the United States, in which the adults are readily identifiable by the markings and indentations on the pronotum, elytra, abdominal sclerites, and profemur (Johnson-Cicalese 1990, Shetlar 2011). There are also several characteristics to distinguish the pupal stage of billbug species, primarily setae, the length of the rostrum, and the width of the pronotum (Satterthwait 1931). However, there are currently no published external characteristics to distinguish between the species of white, legless, grub-like billbug larvae (Vittum et al. 1999). When several species co-occur, as is common with billbugs infesting turfgrass across North America, identification of larvae based solely on association with the presence of the adults may be unreliable. A relatively poor understanding of the seasonal phenology and overwintering behavior of many *Sphenophorus* spp. has constrained the development and implementation of integrated pest management (IPM) programs for the billbug species complex. Other systems have successfully employed molecular methods to associate the adult and larval stages of morphologically cryptic insects (Miller et al. 2005) and multi-species assemblages (Ahrens et al. 2007). Although molecular life-stage association has been investigated for the white grub (Coleoptera: Scarabaeidae) complex in turfgrass (Doskocil et al. 2008), the utility of this approach has not been previously examined for billbugs.

Four species typically infest managed turfgrass the Midwestern United States, *S. parvulus, S. venatus, S. minimus* Hart, and *S. inaequalis* Say (Johnson-Cicalese et al. 1990), but management strategies in the Midwest are still largely been based on the

seasonal ecology of the most common species, *S. parvulus*. *S. venatus* has recently become more abundant and particularly problematic in this region (Richmond 2014). *S. venatus* phenology varies regionally across the U. S., with up to six overlapping generations per year in Florida (Huang and Buss 2009), two overlapping generations per year in North Carolina (Doskocil and Brandenburg 2012), and one generation per year in New Jersey (Johnson-Cicalese et al. 1990) and northwest Arkansas (Young 2002). Although *S. venatus* may be capable of overwintering as larvae, even in the more northerly parts of its range (Doskocil and Brandenburg 2012, Shetlar et al. 2012), billbug larvae cannot presently be identified morphologically to species level. The objectives of this research were to examine billbug species composition and clarify the seasonal phenology of *S. venatus* in Indiana turfgrass systems. We also aimed to determine if *S. venatus* larvae are capable of overwintering in this region of the U.S. In pursuing this goal, we evaluated the utility of three different genetic loci (COI, 18S, and ITS2) for identifying billbug larvae to species level.

2.3 Methods

2.3.1 Adult species composition and seasonal activity

Four locations in Indiana with a history of billbug infestations were selected for monitoring billbug adult species composition: 1) zoysiagrass fairways at Rolling Hills Country Club; 2) Kentucky bluegrass research plots at the William H. Daniel Turfgrass Research and Diagnostic Center, 3) a stand consisting primarily of Kentucky bluegrass at the Purdue University nursery, and 4) a Bermudagrass athletic field at the Purdue University Bimmel Practice Complex. Pitfall traps were constructed of a plastic deli cup

(16 oz.) with holes in the bottom for drainage or linear traps consisting of a polyvinyl chloride (PVC) pipe with a 2.5 cm slit cut lengthwise across the top, a cap fastened at one end, and the other end inserted into a plastic cup with a lid. Traps were surveyed at least once weekly. Grass species, trapping methods, and monitoring dates for each monitoring site are presented in Table 2.1. Adult billbugs were identified to species based on morphological characters described by Vaurie (1951) and Johnson-Cicalese et al. (1990). Males and females were distinguished by the presence of a groove or depression on the metasternum and the first two abdominal sterna (Johnson-Cicalese et al. 1990). Abundances were plotted against Julian date (JD) to describe differences in the seasonal activity of billbug adults in Indiana. To test for sex-biased seasonal activity in *S. venatus*, the number of male vs. female *S. venatus* adults were compared over time using a repeated measures analysis of variance in Statistica 13 (Dell Inc. 2016).

To search for larvae and pupae, ten soil cores (9 cm depth, 10.16 cm diameter), were extracted with a standard golf course cup-cutter and destructively sampled in the field. Soil sampling occurred on a weekly basis April through October in 2009 at Rolling Hills Country Club in Warrick County, IN. Sampling occurred monthly December through February and weekly March through November during 2015 and 2016 at the Bimmel Practice Complex in Tippecanoe County, IN. All larvae and pupae were preserved in 95% ethanol. To characterize larval phenology across the growing season, all larvae collected from the Bimmel Practice Complex in 2015 and 2016 were dorsally imaged using a Leica DFC450 camera mounted onto a MC165C stereomicroscope and head capsule widths were measured using the Leica Application Suite version 4.2.0

(Leica Microsystems, USA) (Figure 2.1). Head capsule width was used as an indicator of larval development.

2.3.2 DNA-based life stage association

Genomic DNA was extracted by following the procedure described in the DNeasy blood and tissue kit (Qiagen, Valencia, CA) from three *S. venatus, S. parvulus, S. minimus,* and *S. inaequalis* adult specimens collected in 2015 or 2016. Genomic DNA from seven larvae collected from Bermudagrss (11 March-7 April 2015, 11-29 March 2016), and three additional larvae collected from Kentucky bluegrass (14-19 July 2016) was also extracted. One *Listronotus maculicollis* was included as an outgroup. Three loci, cytochrome oxidase c subunit 1 (COI), 18S, and the second internal transcribed spacer region (ITS2), covering mitochondrial (mtDNA), ribosomal (rDNA), and nuclear ribosomal (nrDNA) DNA, respectively, were amplified using polymerase chain reaction (PCR) (Table 2.2, Table 2.3). Amplified DNA fragments were confirmed using gel electrophoresis in a 1% agarose gel in TBE buffer. Amplicons were then purified using a PCR purification kit following manufacturer protocols (Qiagen, Valencia, CA).

Purified products were multiplexed by specimen and submitted to the Purdue Genomics Core for sequencing on the Illumina MiSeq platform. The pipeline “Wideseq”, involving Nextera transposon tagging and fragmentation, was used. These products were then processed with dual index/flowcell oligocomplementry adapter extensions. The resulting short read sequences were mapped back to known loci reference sequences with a standard alignment program (Langmead and Salzberg 2012) (Table 2.2). The resultant genomic consensus sequences were visualized and trimmed using Geneious R9 (Kearse

et al. 2012). Phylogenetic analyses were conducted using the default maximum-likelihood settings in RAxML v.8 (1000 bootstrap replicates) (Stamatakis 2006). Pairwise analysis of nucleotide differences, the number of nucleotide differences between species divided by the total number of nucleotides in the concatenated sequence, were calculated in Geneious R9 (Kearse et al. 2012). All outputs were edited and annotated with FigTree v1.2.2 (Rambaut and Drummond 2008).

2.4 Results

2.4.1 Adult species composition and seasonal activity

In this study, 2,079 adult billbugs were collected from four locations. Four species were identified: *S. venatus*, *S. parvulus*, *S. minimus*, and *S. inaequalis* (Table 2.4). *S. venatus* and *S. parvulus* were collected on warm- and cool-season turfgrass, with *S. venatus* being the most abundant species on warm-season turfgrass and *S. parvulus* the most abundant on cool-season turfgrass. *S. parvulus*, *S. minimus*, and *S. inaequalis* were all univoltine with adult abundance peaking at Julian date (JD) 157, 162, and 148, respectively (Figure 2.2). *S. venatus* initiated activity earlier in the spring (JD 85) and remained active throughout the growing season with multiple peaks of adult activity (Figure 2.2). *S. venatus* adults were most abundant at JD 113 (April), with lesser peaks occurring at JD 143 and 150 (May). Three peaks of adult activity were observed consistently across all three trapping seasons: 1) overwintered adults becoming active in the spring, 2) a second peak of late spring activity, possibly resulting from maturation of overwintered larvae, and 3) a broader, less defined peak likely representing a second generation of adults resulting from the two overwintering cohorts (Figures 2.3).

Significantly more male than female *S. venatus* beetles were trapped at the Bimmel Practice Complex ($F_{1, 2}$=25.72, p=0.037). In 2015, 67% of the 489 total *S. venatus* adults trapped at the Bimmel Practice Complex were male. Similarly, in 2016 69% of the 588 *S. venatus* adults trapped were male. This sex ratio was not affected by the time of year ($F_{30,60=}$ 0.64, p=0.909), thus sexes were combined and the weekly proportions of total *S. venatus* adults and *Sphenophorus* larvae were plotted against Julian date to further clarify phenology (Figure 2.3).

Fifty seven larvae were collected from April through October 2009 at Rolling Hills Country Club. Sixty eight and twenty two larvae were collected at the Bimmel Practice Complex from March to December in 2015 and March through November in 2016, respectively (Figure 2.3). Larval head capsule width ranged from 0.73 mm to 2.42 mm (Figure 2.4) and varied across the growing season (Figure 2.5). Larvae with the widest head capsules were observed coming out of winter at JD 85 (March) and again at JD 180 (late June/early July). The smallest mean head capsule widths were observed at JD 160 (early June), likely marking the occurrence of first generation larvae from overwintered adults in the soil. No clear trends emerged for the remainder of the growing season with a mixture of various sized larvae present in the soil after JD 230 (early August) (Figure 2.5). Four *Sphenophorus* pupae were found at the Bimmel Practice Complex in 2015 between Julian dates 192 and 213 (mid-July) and two *Sphenophorus* pupae were found in 2016 between Julian dates 187 and 217. These dates likely correspond with the end of the immature phase of development for the first full generation resulting from overwintered adults and larvae. All larvae and pupae were collected from within 6 cm of the soil surface.

2.4.2 DNA-based life stage association

We obtained sequences from three specimens of *S. venatus*, *S. parvulus*, *S. minimus*, and *S. inaequalis* adults, 10 *Sphenophorus* spp. larvae, and one *Listronotus maculicollis* adult. Maximum-likelihood trees constructed based on only COI consensus sequences resulted in monophyletic clades for *S. venatus* and *S. minimus* (bootstraps>50%) and overwintered larvae were only recovered with *S. venatus* adults (bootstraps>50%) (Figure 2.6). Maximum-likelihood gene trees constructed using only 18S or ITS2 sequences were largely uninformative, with less than six bootstrap values >50% and little recovery of adults of a given species together. The only exception was *S. inaequalis* adults that were all recovered together with only ITS2 (bootstrap=92%) (Figures 2.7 and 2.8).

Based on maximum-likelihood analysis of concatenated COI, 18S, and ITS2, five of the ten unknown larvae were recovered with *S. venatus*, *S. minimus*, and *S. parvulus* adults (bootstrap value>70%). Four additional larvae were recovered with *S. venatus* and *S. parvulus* adults, but had lower support (bootstrap <50%). Only one larva was not recovered with any adults. Of the ten sequenced larvae, five were overwintered. Four overwintered larvae were identified as *S. venatus* (bootstrap>40%) and one was identified as *S. parvulus* (bootstraps>70%) (Figure 2.9). The average pairwise distance similarity of *S. venatus* conspecific adults was high, averaging 99.1% (range, 99-99.3%). The average similarity between larval specimens that were recovered with *S. venatus* adult specimens was 98.7% (range, 94.6-99.5%) (Table 2.5).

2.5 Discussion

Eleven *Sphenophorus* species are pests of managed turfgrass. However, the species composition and abundances of turf-infesting billbugs varies regionally across North America (Dupuy and Ramirez 2015). In Indiana, we trapped four billbug species: *S. venatus*, *S. parvulus*, *S. minimus*, and *S. inaequalis*. These four species have been previously documented in this region of the country based on historical collection data (Johnson-Cicalese et al. 1990). Johnson-Cicalese (et al. 1990) observed nearly equal abundances of these four species in cool-season turfgrasses in New Jersey, while in the present study, *S. venatus* dominated warm-season turfgrasses and *S. parvulus* was most abundant in cool-season Kentucky bluegrass. Dissimilarities in billbug species compositions and abundances between trapping sites has also been documented in South Carolina (Chong 2015), North Carolina (Doskocil & Brandenburg 2012), and Florida (Huang & Buss 2009) and may be related to the host species present, variation in management regimes, or a combination of these factors.

Three of the four species, *S. parvulus*, *S. minimus*, and *S. inaequalis*, all produced one generation a year, and had significant overlap in peak adult activity from May-June. In contrast, *S. venatus* adults initiated activity earlier in the spring (March) and displayed multiple peaks of adult activity throughout the growing season. Important differences in the seasonal activity of *S. venatus* relative to the three additional sympatric billbug species documented during this study could complicate management in regions where these phenologically divergent species are present. These findings emphasize the importance of proper monitoring, identification, and knowledge of billbug seasonal biology as prerequisites for sound insecticide programming.

This is the first study investigating hunting billbug *S. venatus* seasonal biology in the Midwest. As in the present study, male-biased trap captures for *S. venatus* across the growing season has been reported in New Jersey (Johnson-Cicalese et al. 1990), Florida (Huang and Buss 2009), and South Carolina (Chong 2015). The apparently male-biased sex ratio observed in the present study, (67% to 69% male), was most similar to findings of Johnson-Cicalese et al. (1990) in New Jersey, where 65% of *S. venatus* adults were male. However, Young (2002) reported a male-biased sex ratio for only a short period early in the spring, not across the entire year. Because pitfall trapping is a passive collection technique, conclusions about the sex ratio of *S. venatus* populations could indicate that males are more mobile or more active than females, not necessarily that there are more males in the population. The development of dependable, less passive sampling techniques, such as those involving semiochemicals, may be required to confidently characterize the sex ratio of *S. venatus* populations and determine how sex ratio might influence seasonal population dynamics and management options.

The discovery of overwintered adults and larvae in March and April followed by three to four overlapping peaks of adult and larval activity is indicative of two separate, but overlapping, cohorts. Overwintered larvae immediately resume feeding on plant crowns, roots, stolons, and rhizomes in the spring, eventually emerging as adults during late spring and early summer. This pattern has been previously observed in cool-season turfgrass in New Jersey (Johnson-Cicalese et al. 1990), as well as warm-season turfgrass in Arkansas (Young 2002), Virginia (Chong 2015), and North Carolina (Reynolds et al. 2015). Preventive application of plant systemic insecticides targeting both adults and overwintered larvae may provide the most suitable strategy for managing billbug

populations when *S. venatus* present, but this strategy should be explicitly examined. A second application of insecticide 6-8 weeks after the first application may be required due to the occurrence of two *S. venatus* cohorts and the presence of other sympatric species in this region (Richmond 2016). Monitoring of adult activity with pitfall traps or actively scouting the turfgrass surface and nearby pavement areas to determine when adults first become active in the spring should be employed to accurately time insecticide applications.

Head capsule widths from field-collected *Sphenophorus* larvae ranged from 0.733 mm to 2.420 mm. Similar to other weevil species, billbugs have five larval instars (Dupuy and Ramirez 2016), but the relationship between larval size and developmental instar has not been established. In North Carolina, field-collected *Sphenophorus* spp. larvae were grouped into three size classes: small (head capsule width <1.0 mm), medium (head capsule width between 1.0 and 1.7 mm), and large (head capsule width >1.7 mm) (Doskocil and Brandenburg 2012). Medium sized larvae were present across almost the entire year, while small and large larvae were primarily present during two different periods; May-August and September-October for small larvae, and February-April and July-September for large larvae. Since a histogram of observed head capsule widths generated in the present study did not provide the level of resolution necessary to clearly delineate the different larval instars sizes, larvae were divided into three size classes: small (head capsule width <1.2 mm), medium (head capsule width between 1.2 mm and 1.8 mm), and large (head capsule width >2.0 mm), similar to the method employed by Doskocil and Brandenburg (2012). These groupings provided a relatively coarse, but useful, basis for interpreting larval phenology during the growing season. Small larvae

were present in the soil during two distinct periods, May-June and August-November. The first appearance of small larvae in the soil in May likely marks the first generation of larvae resulting from overwintered adults and a potential target for insecticide applications. Although all three size classes were present in the soil in December, only medium and large larvae were found in March of the following year. It is unclear if this finding indicates that smaller larvae are unable to successfully overwinter at the more northerly latitude of Tippecanoe County, Indiana, or if some development continued after sampling was stopped for the season.

The lack of known morphological characters to distinguish billbug larvae to species leaves the seasonal dynamics and differences in overwintering behavior of billbug larvae largely unresolved. This study was the first attempt, to our knowledge, to use molecular methods to associate billbug adults and larvae. Based on maximum-likelihood analyses, the three genetic loci utilized (CO1, 18S and ITS2) varied in their utility for distinguishing the four billbug species examined. Analysis of concatenated sequences from all three loci and COI by itself resulted in four overwintered larvae that were consistently recovered with *S. venatus* adults as a monophyletic clade, providing the first direct evidence of *S. venatus* larvae overwintering above 40°N latitude.

Maximum-likelihood analyses of concatenated sequences and COI recovered a highly-supported clade with all three *S. minimus* adult specimens and two larvae. The two larval specimens that were recovered as *S. minimus* were collected June 2016 from Kentucky bluegrass research plots at the Purdue Nursery where *S. parvulus* was the most abundant adult species (Table 2.4). These results emphasizes that identification of billbug larvae based solely on association with the most abundant adult species is unreliable

where several species co-exist and stresses the necessity for continued development of a billbug larval identification tool.

S. parvulus and *S. inaequalis* adults, although morphologically distinct, were not able to be separated based on the sequences examined in the present study. However, analysis of concatenated sequences (COI, 18S, and ITS2) and COI by itself provided high support for one larva collected from Kentucky bluegrass to be recovered with a *S. parvulus* adult. In analysis of concatenated sequences from all three loci, one overwintered larva collected from Bermudagrass was recovered with a *S. parvulus* adult, but this relationship was inconsistent. *S. parvulus* is not known to overwinter in the larval stage (Dupuy and Ramirez 2015) and *S. parvulus* adults were not recovered as a distinct monophyletic group in any of the analyses. In addition, pairwise distance analyses indicated only 93.28% similarity between the associated overwintered larva and *S. parvulus* adult, which was less than its similarity with the monophyletic clade of *S. venatus* adults and recovered *S. venatus* larvae (>95% similarity). The low similarity of this particular larval specimen with all of the specimens included in our analysis does not conclusively support its identification as any of the target taxa.

This work provides a foundation for the development of a molecular tool useful for identifying billbug larvae but further work will be necessary to refine this technique as a dependable diagnostic tool. A broader taxon sampling covering a larger geographical region is necessary. Specimens from this study were sampled from a small geographical region in Indiana and larvae could not be reared to adults in the lab to confirm molecular analysis. In addition, maximum likelihood analyses were conducted under the default settings in RAxML. Incorporation of gene-specific evolutionary rate models may

increase support and provide more resolution, especially for analysis of concatenated sequences. Taxon-specific PCR primers should also be developed and inclusion of additional loci may increase resolution and establish more consistent relationships for the molecular identification of billbug larvae.

This is the first study to investigate *S. venatus* adult seasonal biology in the Midwestern U.S. and the first to provide molecular confirmation that *S. venatus* is capable of overwintering in the larval stage. Results show that *S. venatus* overwinters in Indiana as both adults and larvae, resulting in two separate cohorts during the spring, each producing at least one subsequent generation of larvae and adults during the remainder of the growing season. The presence of two separate, over-lapping *S. venatus* cohorts presents obvious management challenges. Although an assortment of synthetic insecticide products with extended residual activity are currently available, proper timing will likely be paramount in order to reduce the number of applications required. A combination of cultural, biological, and chemical management strategies that include contact or plant-systemic insecticides targeting adult and larval stages during the spring should be evaluated. Results of the present study also provide a foundation for future work concentrating on the development of a molecular tool to associate billbug life stages. Such a tool will be useful for investigating larval phenology across North America where different assemblages of sympatric billbug species occur.

Table 2.1 Field site, grass species surveyed, pitfall trap design, and monitoring dates for billbug species composition survey in Indiana (2009; 2014-2016).

Field site	IN County	Grass Species	Trapping method	Dates
Rolling Hills Country Club	Warrick	zoysiagrass	Linear pitfall	April-October 2009
Daniel Turfgrass Research & Diagnostic Center	Tippecanoe	Kentucky bluegrass	Cup pitfalls	May-August 2014; March-October 2015, March-August 2016
Purdue University Nursery	Tippecanoe	Kentucky bluegrass	Linear pitfalls	March-August 2016
Purdue University Bimmel Practice Center	Tippecanoe	Bermudagrass	Cup pitfalls	May-August 2014; March-October 2015 and 2016

Table 2.2 Primers for PCR amplification of COI, 18S, and ITS2 from *Sphenophorus* spp. larvae (n=10) and *S. venatus*, *S. minimus*, *S. inaequalis*, and *S. parvulus* adults (n=3 for each species) for DNA-based life-stage association. Taxa and GenBank accession numbers for reference sequences used in the "Wide-Seq" pipeline at the Purdue Genomics Core Facility.

Gene	Primer Sequence[a]	Reference taxa	Accession #
CO1	(F)TAATACGACTCACTATAGGGCAACATTTATTTTGATTTTTTGG (R)ATTAACCCTCACTAAAGTCCAATGCACTAATCTGCCATATTA	*S. venatus*	AF131117
18S	(F)TACCTGGTTGATCCTGCCAGTAG (R)GACGGTCCAACAATTTCACC	*Sitophilus granarius*	AF389038
ITS2	(F)AATACGACTCACTATAGGGTGAACATCGACATTTYGAACGCACA (R)TTAACCCTCACTAAAGTTCTTTTCCSCTTAYTRATATGCTTAA	*Miarus graminis*	AY837713

[a] Forward (F) and reverse (R) primer sequence fragments from Cline et al. (2014).

Table 2.3 Amplification conditions for PCR reactions.

Gene	Hot Start °C (min)	Denature °C (min)	Anneal °C (min)	Extend °C (min)	Final Extend °C (min)	Cycles
CO1	94 (2:00)	94 (1:00)	48 (1:00)	72 (1:00)	72 (12:00)	40
18S	95 (10:00)	94 (0:30)	50-55 (0:30)	72 (1:30)	72 (10:00)	41
ITS2	95 (5:00)	95 (1:00)	57-60 (0:30)	72 (1:00)	72 (7:00)	33

Table 2.4 Billbug species abundances by location. Grass types and years surveyed are indicated in parentheses.

Species	Location				Total
	Rolling Hills CC (zoysiagrass; 2009)	Bimmel Center (Bermudagrass ; 2014-2016)	Daniel Center (KYB[a]; 2014-2015)	Nursery (KYB; 2016)	
S. venatus	116	1482	15	0	1613
S. parvulus	3	49	214	136	402
S. minimus	0	1	24	31	56
S. inaequalis	0	2	2	4	8
Total	119	1534	255	171	2079

[a] KYB, Kentucky bluegrass

Table 2.5 Pairwise distances between taxa expressed as a percentage of nucleotide similarities (COI, 18S, ITS2). Values ≥99% are indicated in bold.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
S. parvulus (1)																						
S. parvulus (2)	92.3																					
S. parvulus (3)	87.9	92.7																				
S. venatus (1)	92.7	98.9	92.8																			
S. venatus (2)	92.6	98.8	92.6	**99.3**																		
S. venatus (3)	92.2	98.9	93.1	**99**	**99.1**																	
S. inaequalis (1)	89.6	94.8	95.3	94.4	94.6	94.7																
S. inaequalis (2)	88.2	93.7	94.7	93.5	93.6	93.7	98.1															
S. inequalis (3)	88.9	95.9	94.1	95.3	95.2	95.5	96.6	95.5														
S. minimus (1)	88.6	93.1	90.5	93.4	93.4	93.2	90.3	90.6	89.7													
S. minimus (2)	87.3	93.4	92.3	93.1	92.9	93.4	91.9	92.2	92.5	96.9												
S. minmus (3)	88.5	93.6	91	93.5	93.4	93.8	90.7	91	90.3	**99.1**	97.4											
Listronotus sp.	84.4	88.4	83.1	87.7	87.8	88	84.6	83.5	85.5	83.5	83.6	84										
Larva01	94.8	95.6	89.3	95.7	95.6	95.3	91.6	90.2	92.1	90.1	89.7	90.1	88									
Larva02	91.1	98.2	93.8	98.7	98.4	98.3	96.3	95	98.1	91.5	92.6	91.8	87	94.3								
Larva03	92.5	92.8	92.8	**99.5**	**99.1**	**99**	94.5	93.6	95.3	93.4	93.1	93.5	87.8	95.6	98.8							
Larva04	92.2	98.9	93	**99.3**	98.8	**99.1**	94.6	93.7	95.7	93	93.2	93.7	87.9	95.2	98.7	**99.5**						
Larva05	83.0	86.1	82.5	85.7	85.7	85.9	84.6	83.4	85.1	80.1	82.6	80.8	78	85.6	84.4	85.7	85.9					
Larva06	92.2	98.9	93.6	98.9	94.6	**99.5**	94.7	98.1	96.6	90.3	91.9	90.7	84.6	95.3	98.4	**99**	94.6	86.1				
Larva07	92.5	98.9	92.9	**99**	**99**	98.7	94.7	93.8	95.3	93.5	93.1	93.4	88.2	96.1	98.2	98.9	93	86.4	**99**			
Larva08	89.3	93.7	95.1	94.3	94.2	94.1	91.4	91.2	90.4	93.6	92.5	93.6	83.8	90.7	92.4	94.2	93.9	80.8	93.9	94.1		
Larva09	88.7	93.3	90.8	93.6	93.5	93.5	90.5	90.7	89.9	**99.3**	97.3	87.4	83.4	90.2	91.7	93.5	93.3	80.3	93.2	93.7	93.8	
Larva10	88.5	93.6	91	93.5	93.4	93.8	90.8	91	90.2	**99.1**	97.5	**99.6**	83.7	90.1	91.7	93.5	93.6	80.6	93.5	93.5	93.6	**99.4**

A, *S. parvulus* (1); B, *S. parvulus* (2); C, *S. parvulus* (3); D, *S. venatus* (1); E, *S. venatus* (2); F, *S. venatus* (3); G, *S. inaequalis* (1); H, *S. inaequalis* (2); I, *S. inaequalis* (3); J, *S. minimus* (1); K *S. minimus* (2); L, *S. minimus* (3); M, *Listronotus* sp.; N, Larva01; O, Larva02; P, Larva03; Q, Larva04; R, Larva05; S, Larva06; T, Larva07; U, Larva08; V, Larva09.

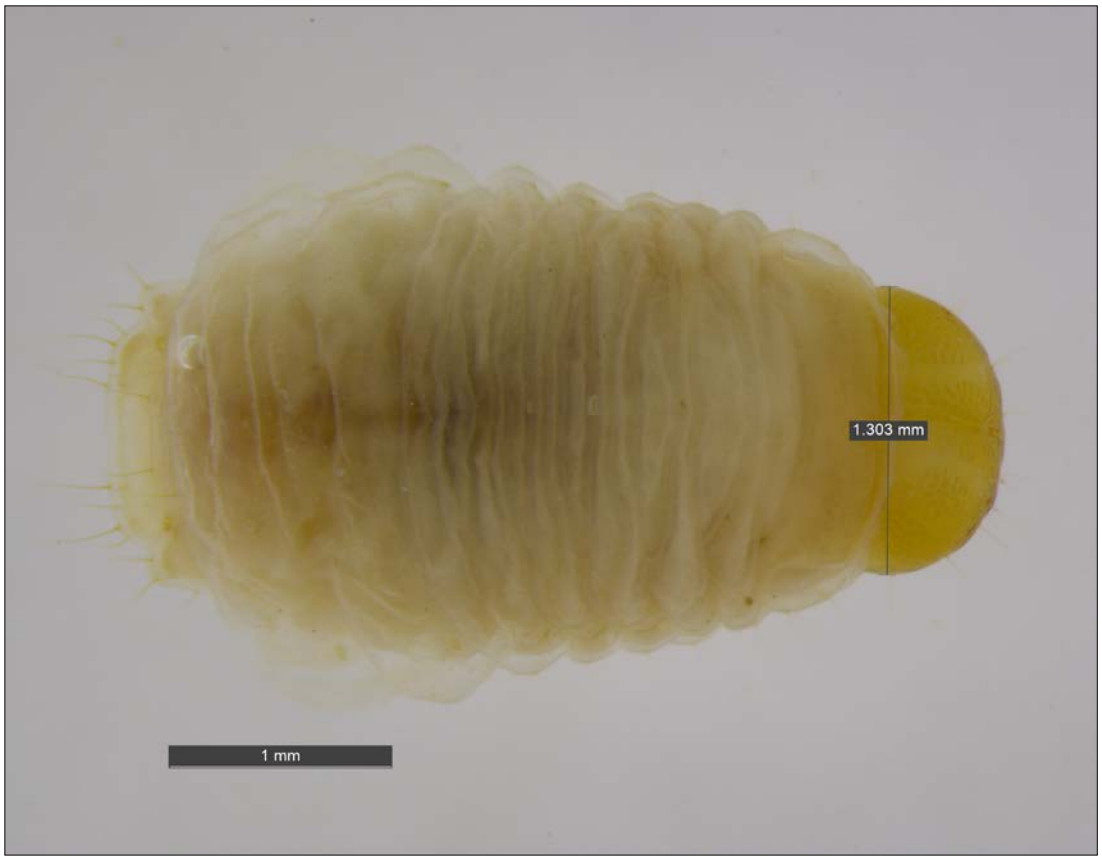


Figure 2.1 Dorsal image of a field-collected *Sphenophorus* larva using a Leica DFC450 camera mounted onto a MC165C stereomicroscope. Head capsule width was measured using the Leica Application Suite version 4.2.0 (Leica Microsystems, USA).

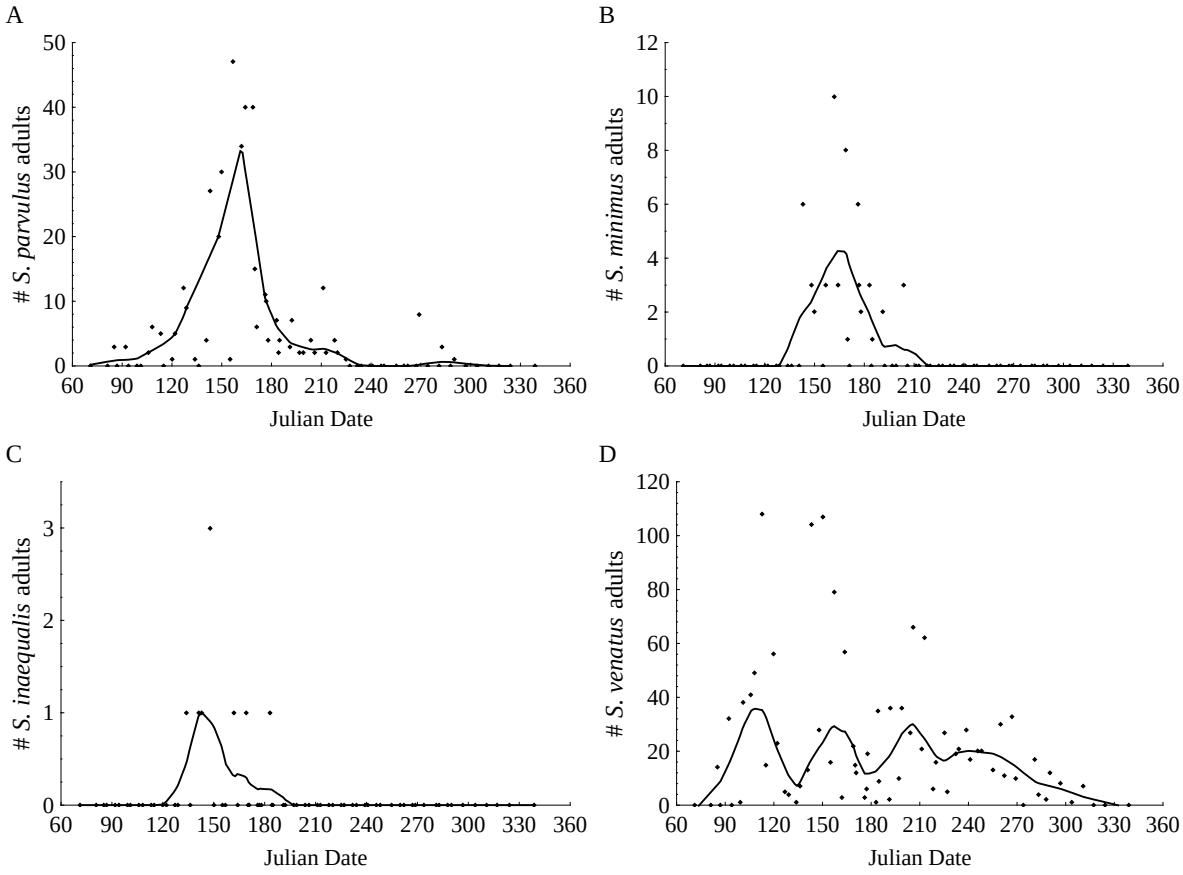


Figure 2.2 Number of adult *Sphenophorus parvulus* (A), *S. minimus* (B), *S. inaequalis* (C), and *S. venatus* (D) adults trapped (2009; 2014-2016). Data were fitted with a robust locally weighted regression (Lowess fit).

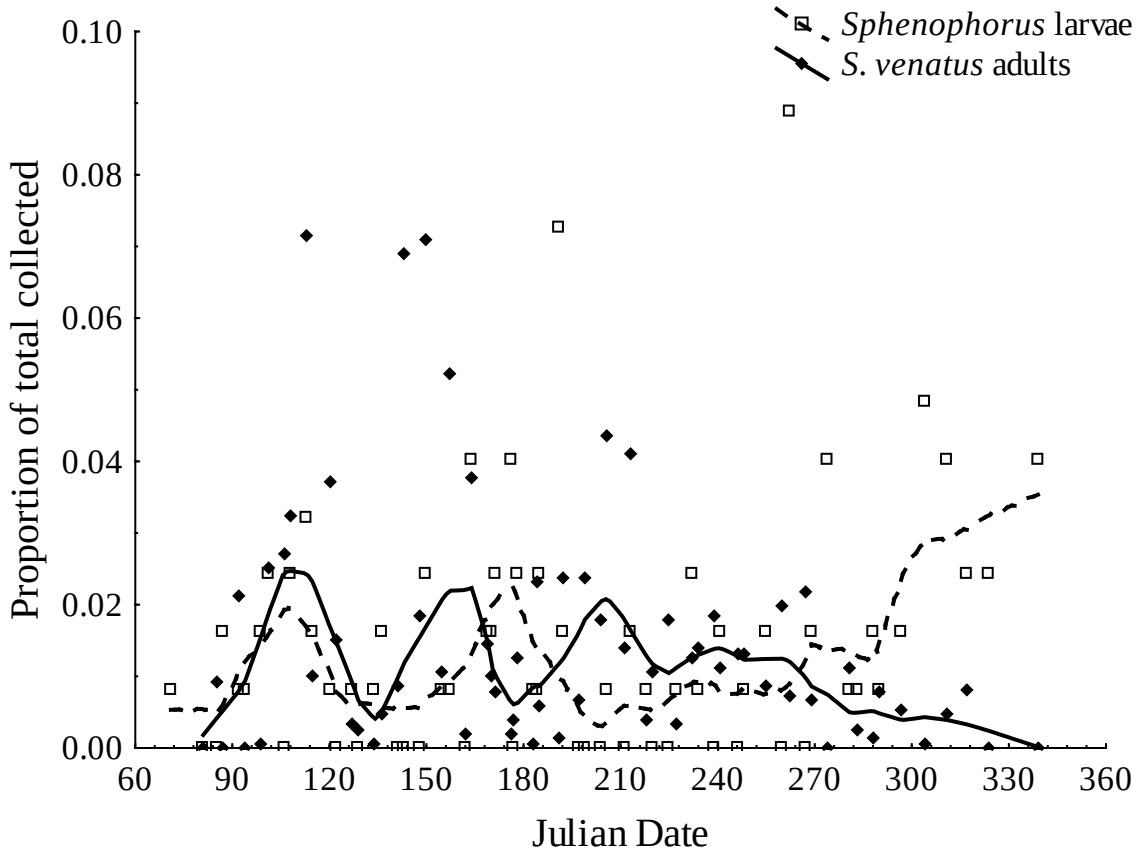


Figure 2.3 Weekly proportions of total *Sphenophorus venatus* adults and *Sphenophorus* larvae collected on warm-season grasses at Rolling Hills Country Club in Newburgh, Indiana (2009) and Bimmel Practice Complex in West Lafayette, Indiana (2015-2016). Data were fitted with a robust locally weighted regression (Lowess fit).

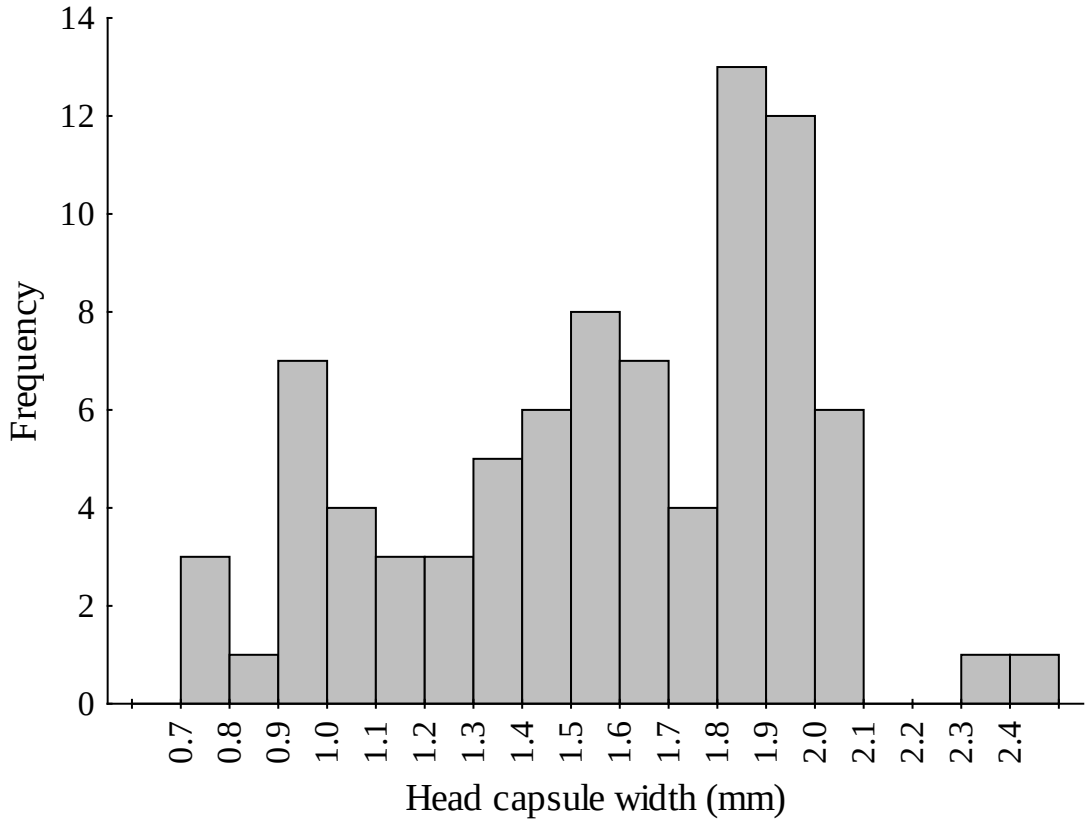


Figure 2.4 Frequency distribution of observed head capsule widths (mm) of field-collected (March-December 2015 and March-November 2016) *Sphenophorus* larvae.

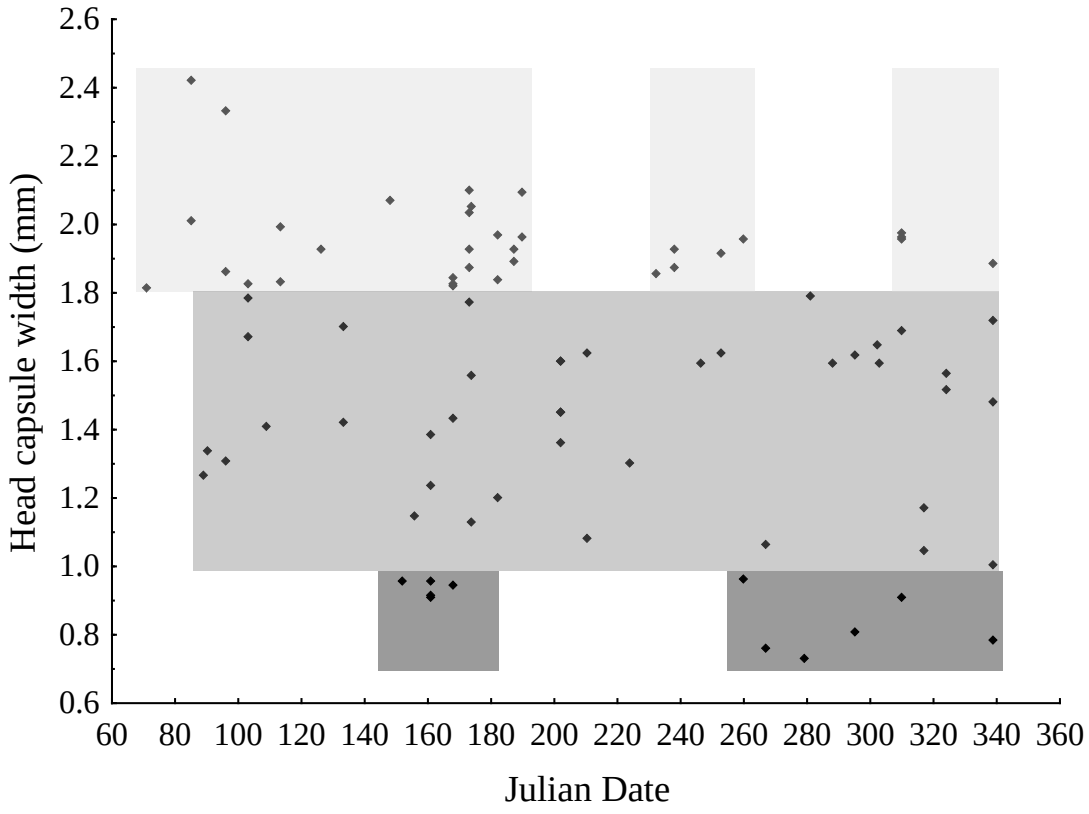


Figure 2.5 Head capsule widths (mm) of all field-collected *Sphenophorus* larvae (n=85) from March 2015-November 2016 at Bimmel Practice Center in West Lafayette, Indiana.

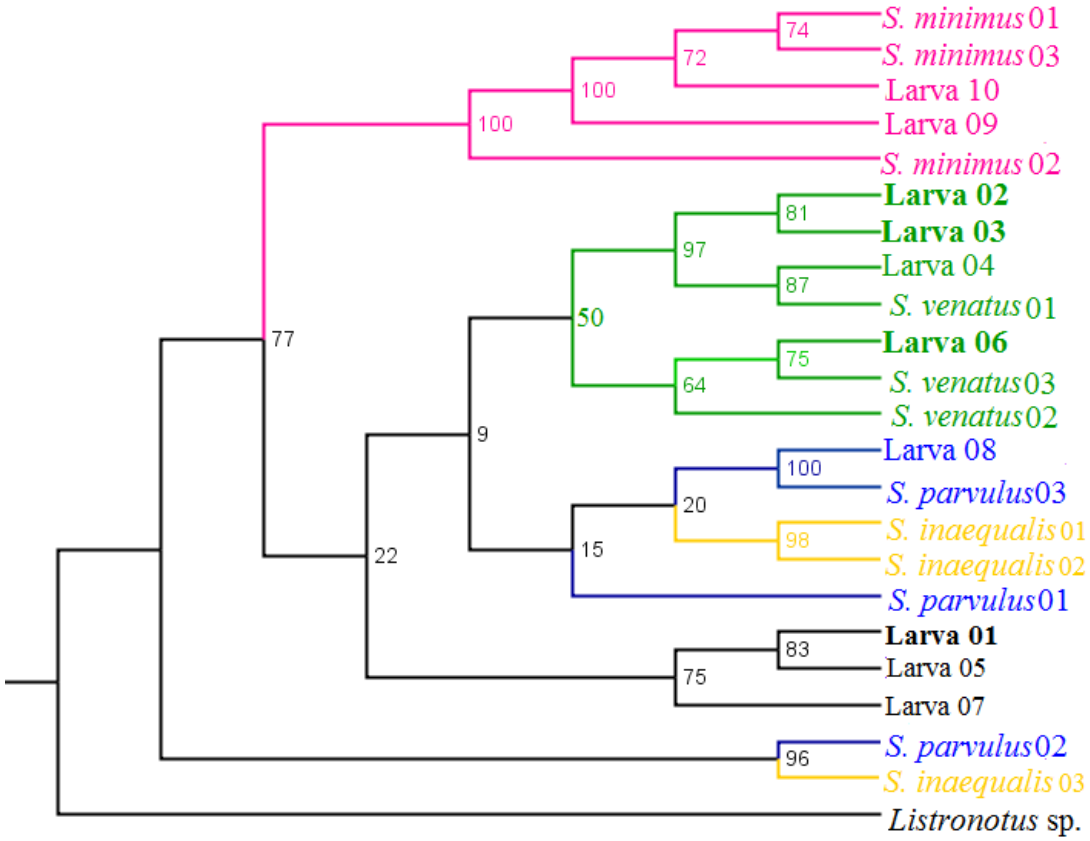


Figure 2.6 Maximum-likelihood tree for COI consensus sequence from *Sphenophorus* spp. larvae (n=10) and *S. venatus*, *S. minimus*, *S. inaequalis*, and *S. parvulus* adults (n=3 for each species). Numbers at nodes are bootstrap values (percentages). Overwintered larvae (larvae collected in March 2015 and 2016) are bolded.

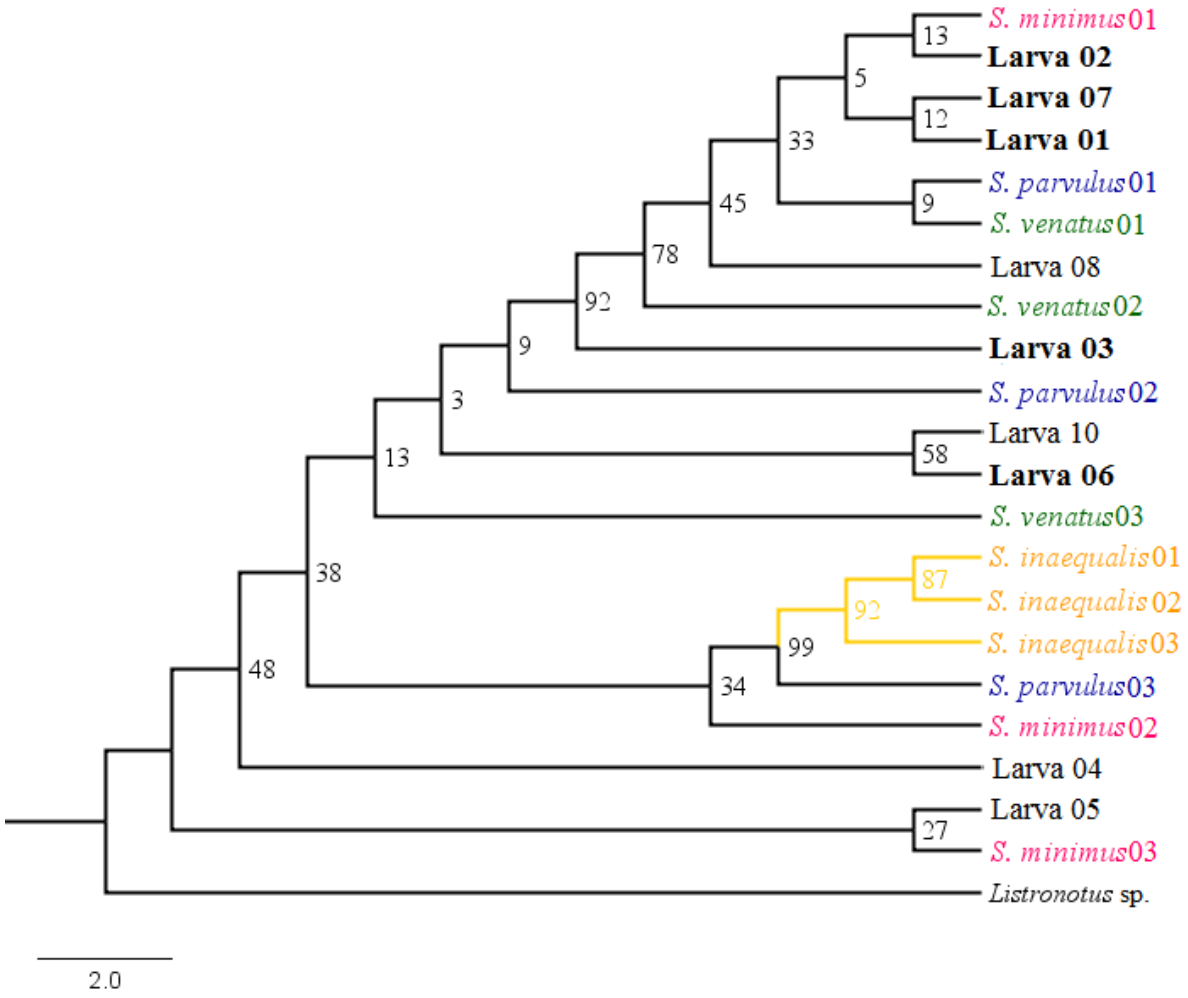


Figure 2.7 Maximum-likelihood tree for ITS2 consensus sequence from *Sphenophorus* spp. larvae (n=10) and *S. venatus*, *S. minimus*, *S. inaequalis*, and *S. parvulus* adults (n=3 for each species). Numbers at nodes are bootstrap values (percentages). Overwintered larvae (larvae collected in March 2015 and 2016) are bolded.

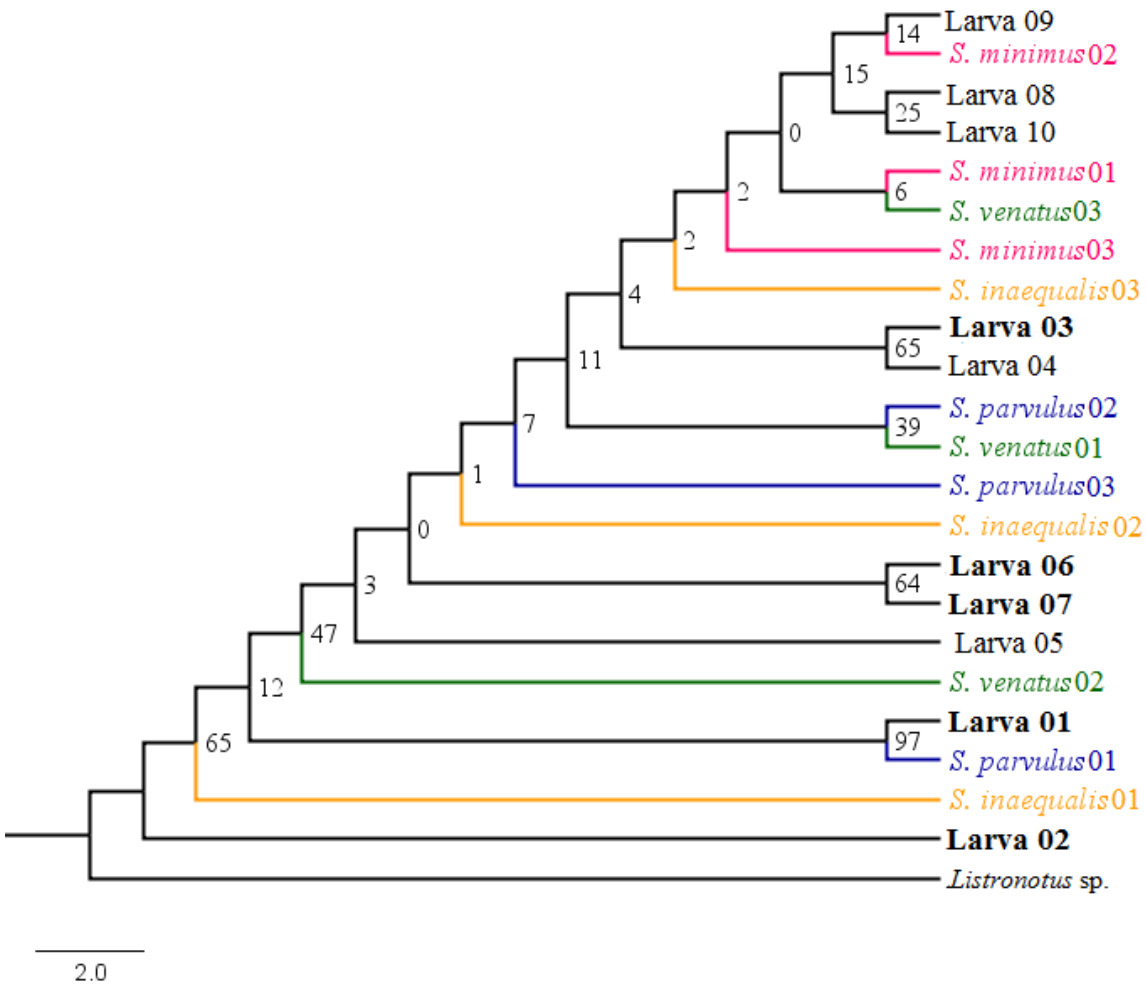


Figure 2.8 Maximum-likelihood tree for 18S consensus sequence from *Sphenophorus* spp. larvae (n=10) and *S. venatus*, *S. minimus*, *S. inaequalis*, and *S. parvulus* adults (n=3 for each species). Numbers at nodes are bootstrap values (percentages). Overwintered larvae (larvae collected in March 2015 and 2016) are bolded.

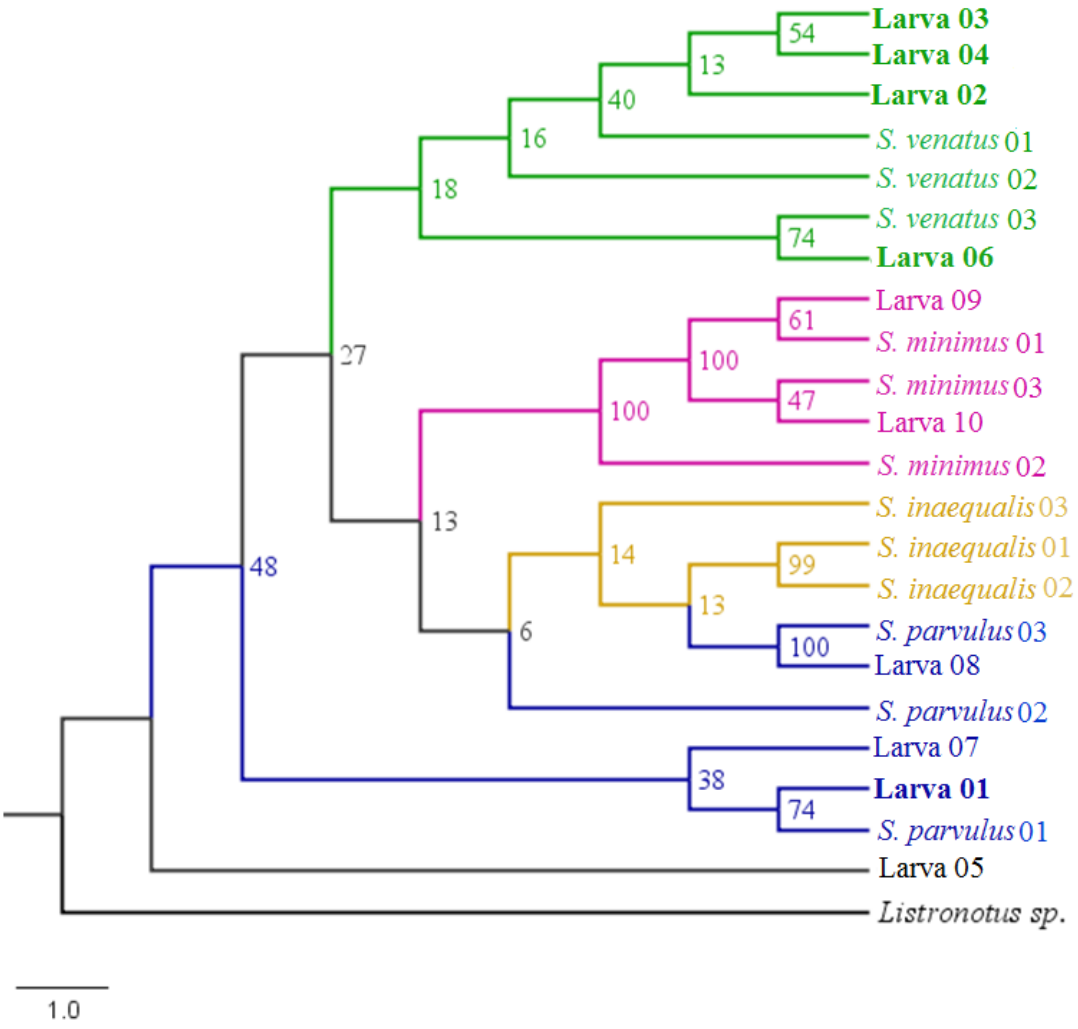


Figure 2.9 Maximum-likelihood tree of COI, 18S, and ITS2 concatenated sequences from *Sphenophorus* spp. larvae (n=10) and *S. venatus*, *S. minimus*, *S. inaequalis*, and *S. parvulus* adults (n=3 for each species). Numbers at nodes are bootstrap values (percentages). Overwintered larvae (larvae collected in March 2015 and 2016) are bolded.

VOLATILE AND CONTACT CHEMICAL CUES ASSOCIATED WITH *Sphenophorus venatus* and *S. parvulus* (COLEOPTERA: CURCULIONOIDEA) HOST- AND MATE-FINDING BEHAVIOR

3.1 Abstract

Beetles in the genus *Sphenophorus* Schönherr, collectively known as billbugs, are native to North America and Europe, where they are historically associated with a diverse assortment of sedges and grasses. Several sympatric species are commonly associated with managed turfgrass, with two species, *S. venatus* Say and *S. parvulus* Gyllenhaal, largely considered the most important pests. This study tested the hypothesis that adult billbugs use volatile organic compounds associated with host-plants and conspecific beetles to direct dispersal behavior. Because mating behavior for these sympatric beetles could potentially be mediated by close-range contact chemical cues, we tested the hypothesis that *S. venatus* Say and *S. parvulus* Gyllenhaal would have qualitatively different cuticular hydrocarbon profiles to facilitate mate-recognition. Field evaluations of the male-produced aggregation pheromone (2-methyl-4-octanol) identified from closely-related congeners (*S. levis* Vaurie and *S. incurrens* Gyllenhaal) did not support the hypothesis that billbug species associated with turfgrass were attracted to this compound. However, in y-tube olfactometer bioassays, *S. venatus* males were attracted to a combination of conspecifics and Bermudagrass *Cynodon dactylon* host-plant material, as well as Bermudagrass host-plant alone. *S. venatus* females were attracted to a combination of male conspecifics and host plant material and male conspecifics alone.

Gas chromatography-mass spectrometry (GC-MS) analysis of *S. venatus* and *S. parvulus* whole-body cuticular extracts indicated series of aliphatic hydrocarbons with qualitative and quantitative interspecific differences as well as intraspecific quantitative differences between males and females. This study provides the first direct evidence that long-range volatile chemical cues direct dispersal behavior of billbugs associated with turfgrass. Findings also substantiate the presence of cuticular hydrocarbons that could serve an important role as contact pheromones for sympatric *Sphenophorus* species.

3.2 Introduction

Behavior-modifying chemicals, or semiochemicals, influence insect behavior at various spatial scales. Long-range semiochemicals such as host-plant volatile organic compounds (VOCs), volatile sex pheromones, or aggregation pheromones, are typically olfactory-perceived, highly-volatile attractive stimuli that elicit upwind orientation and approach to the host, potential mate, or conspecifics. In contrast, close-range semiochemicals associated with mating behavior are typically non-volatile, contact or gustatory-perceived stimuli composed of long-chain hydrocarbons within the epicuticular wax layer (Thornhill & Alcock 1983). These contact pheromones often serve as species- and sex-specific recognition cues. The use of both volatile and contact semiochemicals sequentially for successful mating has been documented other insect species (Guarina et al. 2008, Hughes et al. 2015, Eliyahu 2008).

Within Dryophthoridae (Coleoptera: Curculionoidea), simple branched secondary alcohols act as volatile, male-produced aggregation pheromones for *Sitophilus* Schönherr (Schmuff et al. 1984), *Rhynchophorus* Herbst (Giblin-Davis et al. 1996), *Scyphophorus*

Schönherr (Ruiz-Montiel et al. 2003), and *Sphenophorus* Schönherr (Zarbin et al. 2003) species. Cross-attraction of sympatric weevils has been reported for many of these synthetic pheromone lures and racemic mixtures, suggesting that the majority of aggregation pheromones in the Rhynchophorinae are not species-specific (Francke and Dettner 2005, Giblin-Davis 1996). Long-range volatile aggregation pheromones that display significant interspecific activity may require additional close-range, tactile chemical cues, such as cuticular hydrocarbons, to facilitate intraspecific interactions.

Not surprisingly, cuticular hydrocarbons (CHCs) act as contact pheromones in multiple Curculionoid species (Lapointe et al. 2004, Pomonis and Hakk 1984, Martins et al. 2013, Mutis et al. 2009). These weevils displayed behaviors commonly associated with insects that rely on contact pheromones, such as antennal and/or tarsi contact prior to mounting and copulation. Despite sympatric species co-occurring across multiple genera in Dryophthoridae, most studies have focused on sex-specific compounds or differences in the relative abundances between males and females of a single species; only one study has previously documented the presence of close-range, species-specific recognition cues for multiple closely-related weevil species (Baker et al. 1984).

Billbugs, a group of stem-boring beetles in the genus *Sphenophorus* Schönherr (Coleoptera: Dryophthoridae), are associated with a diverse assortment of grasses and sedges (Vaurie 1951). Over 60 species of *Sphenophorus* are native to North America, with at least ten species being pests of managed turfgrass (Dupuy and Ramirez 2016). Of these, the hunting billbug *S. venatus* Say and bluegrass billbug *S. parvulus* Gyllenhaal are the most widely distributed and economically important species (Johnson-Cicalese 1990, Vittum et al. 1999). Adults disperse from overwintering sites in the spring to nearby

turfgrass stands where they mate and lay eggs in the grass stems. Larvae damage plants by feeding on or inside the stems, crowns, roots, stolons, and rhizomes. Damage first appears as areas of brown, dying grass about 5-8” in diameter, sometimes forming larger, irregular patches. Billbug damage is widely misdiagnosed, with symptoms often being confused with disease, drought, or nutrient deficiency. Such misdiagnosis can lead to weed encroachment or unnecessary and ineffective herbicide and fungicide inputs (Vittum et al. 1999).

Chemical intervention is often the only viable option to manage billbug pests in turfgrass and three basic strategies, targeting different life stages, have been most widely adopted (Richmond 2016, Shetlar and Andon 2012): 1) preventative control of adults using contact insecticides when adult activity is initiated in the spring 2) preventive control of early instar larvae inside the stems using systemically active insecticides and 3) curative application of soil insecticides targeting larger larvae in the soil after damage is visible. To reduce reliance on chemical insecticides and improve management, billbug research in recent decades has focused on understanding regional variation in species composition (Chong 2015, Huang and Buss 2009, Johnson-Cicalese et al. 1990) and seasonal activity (Doskocil and Brandenburg 2012, Young 2002), improving insecticide application timing (Reynolds and Brandenburg 2015), and developing effective cultural management strategies (Huang and Buss 2013, Fry and Cloyd 2011, Reinert et al. 2011, Richmond et al. 2000). Billbug chemically-mediated behavior and the potential for incorporating semiochemicals into billbug monitoring and management programs has not been previously studied.

Synthetic sex pheromones are available for monitoring flights of several Lepidoptera (Alm et al. 1999) and Scarabaeid (Alm et al. 2006, Potter and Haynes 1993, Robins et al. 2009) pests of turfgrass. Unfortunately, the relatively strong flight and dispersal capacity of these insects makes the relationship between the number of adults captured and subsequent local pest densities weak and of limited utility for making pest management decisions (Potter and Haynes 1993). Conversely, monitoring and control approaches involving semiochemicals have the greatest potential when the adult and larval stages both share the same habitat or when the adults tend to mate and lay eggs on the same site; billbugs fit both of these criteria (Young 2002, Potter and Haynes 1993). Previous work with another stem-boring turfgrass pest in the same superfamily (Curculionoidea), the annual bluegrass weevil *Listronotus maculicollis* Kirby, demonstrated that adults display both behavioral and electroantennographic responses to *Poa annua* host-plant volatiles (McGraw et al. 2011). Like the annual bluegrass weevil, billbugs are not capable of sustained flight but rather walk as their main means of dispersal, suggesting VOCs could also orient adult billbug dispersal behavior. Additionally, multiple, closely-related billbug pest species overlap geographically and temporally. Therefore, once aggregated, the ability of these insects to self-assort and identify suitable mates could hinge on the use of contact chemical cues, such as cuticular contact pheromones. *S. venatus* mating activity mostly occurs between midnight and four A.M. (Huang and Buss 2009) but the mating behavior sequence prior to copulation has not been previously recorded. Preliminary observations of antennating behavior and the common occurrence of multiple sympatric species in turfgrass environments led us to

hypothesize that these turf-inhabiting billbugs may rely on contact pheromones for successful mate-recognition.

This study explored the potential for two forms of chemoreception to mediate billbug behavior: recognition of VOCs and non-volatile cuticular wax components. First, we examined the attractiveness of a known *Sphenophorus* aggregation pheromone, 2-methyl-4-octanol, to *S. venatus* and *S. parvulus* under field conditions during two consecutive growing seasons. Second, we hypothesized that if VOCs influence adult dispersal and orientation, adult *S. venatus* would positively respond to both host-plant material and conspecifics in y-tube olfactometry bioassays. Lastly, we hypothesized that if contact stimuli are used by billbugs to facilitate species and sex recognition, there would be both qualitative and quantitative differences between the cuticular hydrocarbon profiles of *S. venatus* and *S. parvulus*.

3.3 Methods

3.3.1 Insects & plant material

S. venatus adults were collected from Bermudagrass *Cynodon dactylon* L. athletic fields by pitfall trapping or hand-collecting at night from March to October. Adult *S. parvulus* were collected using linear pitfall traps placed in a stand of Kentucky bluegrass *Poa pratensis* L. and hand-collecting on sidewalks from April to October. Billbugs were separated by species and sex and held in glass mason jars covered with tulle fabric containing a moist dental wick, moist paper towels, and host plant material. Glass jars containing billbugs were held in a growth chamber (25-27°C; 78-85% RH; 10:14 (L: D)) until used for bioassays or cuticular extractions.

Plant material for bioassays was obtained by extracting cores of Bermudagrass and Kentucky bluegrass from the field using a standard golf course cup cutter. Plant and soil cores were placed into pots containing potting soil and maintained in a greenhouse until used in bioassays. Grass plants were maintained at a height of 5.0 cm and watered daily.

3.3.2 Pheromone-baited trapping

To determine the extent to which *S. venatus* and *S. parvulus* are attracted to a known synthetic aggregation pheromone (2-methyl-4-octanol) previously identified from two closely-related species, *Sphenophorus levis* Vaurie (Zarbin et. al 2003) and *S. incurrens* (Illescas-Riquelme et al. 2016), three pairs of pitfall traps were monitored at two billbug infested sites in West Lafayette, Indiana during 2014 and one site during 2015. One pitfall trap in each pair contained a pheromone lure comprised of a polyethylene bag holding 20 µl of 2-methyl-4-octanol synthesized by P. Zarbin. Pheromone lures were re-baited bi-weekly. The second trap was positioned 3 meters away and contained an empty bag as a control. The identity of baited or non-baited traps was randomly assigned, but switched weekly to account for predominant wind direction. Traps were monitored weekly from May to June in 2014 and April to July in 2015. The number of individuals from each species and sex of all captured billbugs was recorded.

3.3.3 Y-tube olfactometer bioassays

The response of *S. venatus* adults to five different odor treatments was tested using a y-tube olfactometer (8cm and 12cm arms; 2 cm diameter, round glass joints;

Analytical Research Systems, Gainesville, FL) (Figure 3.1) during peak adult activity periods (April-June and September-October) in 2015 and 2016. Treatments were comprised of five combinations of conspecific beetles and above-ground Bermudagrass *Cynodon dactylon* host-plant material that were all compared to a purified air control: 30 conspecific males + 5g grass, 5g grass, 30 conspecific males, 30 conspecific females + 5g grass, and 30 conspecific females. Purified air was pushed through an activated charcoal filter via copper tubing and split into two air streams held at ~1 liter/min. Each air stream was delivered via Tygon tubing through one of the 8 cm y-tube arms via glass tubes (14cm long, 2 cm diameter) that held odor source treatments. Glassware was washed using 1% diluted Alconox soap, rinsed with acetone, and baked in an oven for ~10 minutes at 200°C between odor treatments, switching sex, or after 30 minutes.

All observations were made in the dark using a red headlamp under laboratory conditions. Billbugs were placed individually at the bottom of the y-tube and observed for a maximum of 10 minutes. Billbugs not making a choice by 10 minutes were recorded as “not responding”. A response was recorded if the billbug walked upwind in the 12 cm arm, 2 cm into the 8 cm arm corresponding with an odor source, and remained there for one minute. Treatments were replicated until 30 male and 30 female responders were observed. Each individual insect was used only once. After each replicate, treatment arms were switched to exclude directional bias.

3.3.4 Preparation of whole-body cuticular extracts

To remove cuticular wax compounds, 10 male and 10 female replicates comprised of five individuals were subjected to two separate, successive washes in analytical-grade

hexane (Avantor Performance Materials, Center Valley, PA, USA). For each wash, five individuals were placed in a 4-ml vial and immersed in 0.5 ml of hexane, vortexed for two minutes, and then placed in a sonication bath for two minutes. For the first wash, insects were removed from the solvent with forceps and placed in a new 4-ml vial containing hexane while the vial was in the sonication bath. This step was preformed to reduce the amount of hydrocarbons adhering to the cuticle. The two washes were then combined and condensed to 0.5 ml under nitrogen. Samples were stored in a freezer (-20°C) until analyzed.

3.3.5 Analysis of cuticular extracts and identification of cuticular hydrocarbons

Samples were analyzed by coupled gas chromatography-mass spectrometry (GC–MS) with electron impact ionization (EI, 70 eV) using a Hewlett-Packard (HP) 6890N gas chromatograph (Hewlett-Packard, Sunnyvale, CA, USA) equipped with a DB-5MS capillary column (30 m X 0.25 mm X 0.25 µm film, J&WScientific, Folsom, CA, USA) in splitless mode, and interfaced to a HP 5975N mass selective detector (MSD), with helium as the carrier gas. Prior to analysis, hexane extract samples were returned to room temperature, vortexed 2 minutes, and sonicated 2 minutes to reduce the amount of hydrocarbons adhering to the glass vial. One microliter was injected into the heated GC injection port (250°C). After a 1 min hold at 50°C the oven temperature was ramped to 280°C at 10°C/min with a hold for 20 min at 280°C. Compounds were identified by comparing the retention times and diagnostic ions of compounds with commercial standards. The abundance of each compound was calculated as a percentage of the total

corrected peak area of all hydrocarbons that were consistently present in the total ion chromatograms (ChemStation, Version B.03.01; Hewlett-Packard Corp.).

3.3.6 Statistical Analyses

The numbers of adults for each species and sex captured in pitfall traps associated with the pheromone field trial were square-root transformed prior to statistical analysis to satisfy the homogeneity of variance assumption. To test the main effect of the pheromone treatment, the transformed number of *S. venatus* and *S. parvulus* male and female adults captured in pheromone baited vs. un-baited traps was examined over time using repeated measured analysis of variance. The proportion of responsive billbugs in y-tube olfactometer bioassays was calculated by dividing the total number of billbugs responding to an odor source (N=30) by the total number of billbugs tested for that odor source treatment. Variation in the mean percentage of responsive billbugs was examined using analysis of variance (ANOVA) with odor source and sex serving as independent variables. Subsequently, a Chi-square goodness of fit test was used to test the null hypothesis that the thirty responsive *S. venatus* adults showed no preference for the manipulated odor source treatment vs. purified air control (a response ratio equal to 50:50). To investigate inter- and intraspecific quantitative differences in the cuticular solvent extracts, the effects of species and sex on the mean relative abundances of individual cuticular components were compared using multivariate analysis of variance (MANOVA) and means were separated using a Tukey (HSD) test. All statistical tests were performed using Statistica (Dell Inc. 2016).

3.4 Results

3.4.1 Pheromone-baited trapping

The aggregation pheromone (2-methyl-4-octanol) of congeners, *S. levis* and *S. incurrens* did not have a significant effect on number of males or females of *S. venatus* or *S. parvulus* (2014: $F \leq 0.17$, $df=6$, $p \geq 0.692$) (2015: $F \leq 7.51$, $df=2$ $p \geq 0.111$) captured in pitfall traps during the experiment. Time did not influence the response to pheromone treatment (2014: $F \leq 1.39$, $df=6$, $p \geq 0.251$) (2015: $F \leq 1.29$, $df=2$, $p \geq 0.315$).

3.4.2 Y-tube olfactometer bioassays

Adult *S. venatus* males and females both oriented towards VOCs in y-tube olfactometry bioassays with seventy-three percent of billbugs responding. There was no significant difference in the mean percentage of males (77.18±2.16) vs. females (71.23±5.66) responding in the y-tube olfactometer bioassays within the allotted ten minutes ($F_{1, 4}=2.42$, $p=0.195$) and the odor source opposite of the purified air control had no significant influence on the percentage of billbugs responding ($F_{4, 4}= 4.01$, $p=0.104$). However, the response to different manipulated treatments did vary between males and females (Figure 1). Males positively responded to all three treatments containing Bermudagrass host-plant material (males + grass: $X^2_{(1)}=8.53$, $p=0.003$), (grass: $X^2_{(1)}=6.53$, $p=0.011$), (females + grass: $X^2_{(1)}=4.80$, $p=0.028$), but not to male ($X^2_{(1)}=1.20$, $p=0.273$) or female ($X^2_{(1)}=1.20$, $p=0.2731$) conspecifics alone. In contrast, females positively responded to the two treatments containing male conspecifics (males + grass: $X^2_{(1)}=8.53$, $p=0.003$), (males: $X^2_{(1)}=10.80$, $p=0.001$), but did not orient toward treatments

containing only host-plant material (grass: $X^2_{(1)}$=0.53, p=0.465) or other females (females + grass: $X^2_{(1)}$=1.20, p=0.273), (females: $X^2_{(1)}$=0.13, p=0.715).

3.4.3 Cuticular hydrocarbons

Analyses of *S. venatus* and *S. parvulus* cuticular extracts revealed a series of several long-chain aliphatic hydrocarbons. Retention times and molecular ionization patterns indicated species-specific chemical differences between the hydrocarbon profiles as well as ten compounds that were shared between the two species. Species-specific chemical differences included one compound that was unique to *S. venatus* and five compounds that were unique to *S. parvulus* (Table 3.1, Figure 3.3, 3.4).

Although there were no sex-specific compounds identified in the extracts from *S. venatus* or *S. parvulus*, statistical analyses of the mean relative abundances supported quantitative differences between species and sexes for several compounds (F=12.52, df=17, p<0.001) (Table 3.2, Table 3.3, Figure 3.5, Figure 3.6). Quantitative differences between sexes were observed for three of the five species-specific *S. parvulus* compounds (Table 3.2, Table 3.3, and Figure 3.6). Of the ten cuticular components shared between species, five compounds were present in similar relative abundances, including the hydrocarbon component that was most abundant in both *S. venatus* and *S. parvulus* females, heptacosane (peak 5) (Table 3.2, Table 3.3, Figure 3.3, Figure 3.4). Two shared compounds differed in mean relative abundance between *S. parvulus* males and females, but not *S. venatus*. One compound differed in mean relative abundances between males and females of both species, and one compound was consistently present in higher relative abundance for *S. venatus* than *S. parvulus* (Table 3.2, Table 3.3).

3.5 Discussion

To our knowledge, this is the first study investigating chemically-mediated behavior in billbugs associated with turfgrass. Findings of this study suggest 1) *S. venatus* males are attracted to host-plant volatiles, 2) the presence of a male-produced volatile sex pheromone that is attractive to females for at least one of the most common billbug species associated with managed turfgrass, *S. venatus*, and 3) qualitative and quantitative differences in the cuticular hydrocarbon profiles that could facilitate mating in both of the species examined.

Because billbugs are not strong flyers, directed movement towards VOCs could serve as a more energy efficient strategy than randomly walking to forage and search for mates (Young 2002). The present study addressed this hypothesis from two different angles. First, we examined the extent to which 2-methyl-4-octanol, a known aggregation pheromone for *Sphenophorus incurrens* (Illescas-Riquelme et al. 2016) and *S. levis* (Zarbin et al. 2003) influenced trap catches of *S. venatus* and *S. parvulus* in the field. We expected that 2-methyl-4-octanol would be attractive to *S. venatus* and *S. parvulus* because these species are closely related to *S. incurrens* and *S. levis* and interspecific activity has been previously documented for many synthetic Rhynchophorin pheromone lures (Francke and Dettner 2003). Contrary to our predictions, results from the present study showed 2-methyl-4-octanol alone did not increase trap capture for *S. venatus* or *S. parvulus* adults. However, the pheromone blends for several previously studied Rynchophorine species contain at least two major chemical components (Franke and Dettner 2005). While 2-methyl-4-octanol has been identified as the only aggregation pheromone component for *S. incurrens* and *S. levis*, it has also been identified as only one

of multiple compounds in the pheromone blends for other closely-related species, including *Metamasius hemipterus* L. (Ramirez-Lucas et al. 1996), the Australian population of *Rhabdoscelus obscurus* Boisduval (Giblin-Davis et al. 2000), and *Scyphophorus. Acupunctatus* Gyllenhaal (Ruiz-Montiel et al. 2008). The present study did not examine the attractiveness of 2-methyl-4octanol as part of a ratio-specific pheromone blend and indicates only that under the specific field conditions of the current study, this compound does not seem to be attractive for *S. venatus* or *S. parvulus* by itself.

Synergism between plant volatiles and aggregation pheromones has been widely documented for weevils (Reddy and Guerrero 2004), suggesting the biological activity of the synthetic pheromone lure could require the presence of host-plant volatiles in order to be attractive to *S. venatus* or *S. parvulus*. This phenomenon was observed in *S. incurrens*, with the 2-methyl-4-octanol pheromone lure + sugarcane host-plant material trapping more weevils than sugarcane alone, sugarcane + male conspecifics, and the 2-methyl-4-octanol pheromone lure alone (Illescas-Riquelme et al. 2016). Findings of the present study suggest *S. venatus* and *S. parvulus* are not attracted to 2-methyl-4-octanol, so our next step was to investigate if billbugs recognize VOCs and what VOCs they orient towards.

Our second approach for addressing the role of VOCs in directing the movement of billbug species associated with turfgrass focused on a series of y-tube bioassays with *S. venatus*. Male and female *S. venatus* beetles displayed significant behavioral responses in the y-tube olfactometer, supporting our hypothesis that adults recognize and orient towards VOCs. Further, orientation towards different odor sources varied between sexes. Males positively responded to all three treatments containing Bermudagrass host-plant

material, suggesting that dispersal behavior of males is primarily oriented toward host-plant volatiles. In contrast, females positively responded to both treatments containing male conspecifics, suggesting that dispersal behavior of females may be driven by a male-produced volatile pheromone.

Sex-specific variation in behavioral responses to host-plant volatiles is common across insects (Szendrei and Rodriguez-Saona 2010). Apparently, *S. venatus* is no exception, as findings from the present study suggest male but not female *S. venatus* adults orient towards host-plant VOCs. This finding does somewhat contrast with results from the only other study investigating chemoreception in curculionid pests of turfgrass. Dissimilar to the hunting billbug, annual bluegrass weevil, *Listronotus maculicollis* females responded positively to host plant (*Poa annua* L.) volatiles but males did not. These behavioral differences are likely related to the fact that, unlike billbugs, which display a brief, male-biased increase in activity during the spring (Young 2002), sex ratio bias during spring colonization has not been observed in *L. maculicollis* (McGraw et al. 2011). Further, the positive response of *S. venatus* females to male conspecific treatments suggests the presence of a male-produced sex pheromone in *S. venatus* and provides support for the idea that males of this species are the primary colonizers of food resources. Within Curculionoidae, 18 of the 21 species that are known to utilize pheromone-mediated sex attraction are more attractive when allowed to feed on host-plant material (Landolt 1997). Male attraction to host-plant volatiles is not surprising if it enhances male pheromone production and reproductive success. Our findings suggests that, although *L. maculicollis* and *S. venatus* are similar in respect to their feeding

behavior and subsequent damage symptoms, they differ in their chemosensory and dispersal behavior.

Female attraction to males is more likely to occur if it reduces energy requirements of mate-searching activities (Thornhill and Alcock 1982). Because billbugs are weak fliers and oviposition occurs at adult feeding sites, female attraction to a male pheromone could provide more energetically efficient access to potential mates, oviposition sites, and food resources. In this study, manipulated odor treatments were always compared to a purified air control. This was done in order to avoid volatiles mixing in the notch of the y-tube and to avoid saturation of antennal receptors that can lead to false repellent effects (Szendrei et al. 2009, McGraw et al. 2011). Future work, comparing male responses to "male + grass" and "grass only" treatments by electroantennography or field trapping studies could clarify how the attractiveness of a male-produced aggregation pheromone is influenced by the presence of host-plant material. In addition, head-space volatile collection of the manipulated odor treatments and subsequent electroantennography will aid in identifying which specific host-plant VOCs influence billbug behavior and clarify the absolute configuration of the putative *S. venatus* male pheromone.

Because as many as 11 billbug species may cohabitate turfgrass environments and many curculionid volatile aggregation pheromones display interspecific activity, VOCs are likely not the only form of chemoreception orchestrating billbug behavior. Analyses of cuticular extracts from two sympatric billbugs species (*S. venatus* and *S. parvulus*) indicated qualitative and quantitative interspecific chemical differences in cuticular hydrocarbon profiles and intraspecific quantitative differences between males and

females of both species. These findings, support the idea that cuticular hydrocarbons could play a role in the mate recognition between these closely related species. Of the components identified from both species, n-alkanes or alkenes were observed. This pattern is similar to that reported for the hydrocarbon profiles of other curculionid adults such as *Cylindrocopturus adspersus* LeConte (Pomonis and Hakk 1984), *Diaprepes abbreviatus* Linnaeus (Lapointe et al. 2004), and *Aegorhinus superciliosus* Guérin (Mutis et al. 2009).

Previous studies have documented the ability of males to recognize female cuticular hydrocarbons through mating bioassays with freeze-killed, solvent-washed freeze-killed, and reconstituted freeze-killed females (Ginzel 2003, Mutis et al. 2009). Antennal contact prior to mounting and copulation is a consistent behavior across insects that rely on tactile chemical signals. Preliminary laboratory observations suggest that male billbugs also antennate females before mounting and continue to tap and/or stroke females with antennae and tarsi once mounted, a behavior that has been consistently observed in other curculionids (Martins et al. 2013). In the laboratory, we observed one *S. venatus* male that attempted to mate with a freeze-killed *S. venatus* female. The mated pair was collected in the field on April 29, 2015. The male was held in a separate container with Bermudagrass while the female was freeze-killed and then allowed to thaw for 30 minutes prior to observation of mating behavior in a glass petri dish lined with filter paper on May 4, 2015. The male made antennal contact prior to mounting the female, oriented himself, continually stroked the female with his front and hind tarsi, and then bent the abdomen to extrude the aedeagus. When presented to the same, but hexane-washed, female the next day, the male made antennal contact and mounted, but

dismounted and did not attempt to mate after thirty minutes, implying that recognition signals in the cuticle may have been removed by the solvent. After the female was reconstituted with 400 µl of the cuticular extract, the male made antennal contact, mounted, curved the abdomen, and extruded the aedeagus within two minutes of being presented to the female. Unfortunately, were unable to replicate this observation. Although a greater number of observations will be required to confirm the biological significance of cuticular hydrocarbons in *Sphenophorus*, the differences in cuticular chemical profiles observed in the present study could provide the basis for mate recognition between these sympatric insects.

This is the first study to demonstrate the capacity for chemoreception at different spatial scales in billbugs associated with turfgrass. Orientation to long-range VOCs emitted from host-plants and adult male conspecifics likely mediate dispersal prior to close-range reliance on cuticular hydrocarbons to facilitate mate recognition. Future work should concentrate on identifying the absolute configuration of the putative male pheromone suggested by our results and the potential for synergism with host-plant VOCs. Although this study provides the first preliminary observations of mating behavior prior to copulation, a better understanding of the mating behavior sequence for these insects will be required to clarify the role of unique cuticular hydrocarbon profiles in close-range interactions between and within species. Knowledge coming from these studies could provide the basis for development of sustainable monitoring and management tactics, such as synthetic pheromone lures and cuticular wax components that could be coated on fertilizer granules or other decoys for mating disruption.

Table 3.1 Qualitative differences and identification of cuticular hydrocarbon components in whole-body hexane extracts of female (♀) and male (♂) *Sphenophorus venatus* and *S. parvulus*[a].

Peak #	Retention Time (min)	Hydrocarbon	*S. venatus*		*S. parvulus*		Diagnostic ions[b]
			♀	♂	♀	♂	
1	22.35		+	+	+	+	
2	22.91		+	+	+	+	
3	23.11		+	+	+	+	
4	23.63	C_{27} monoene	–	–	+	+	**378** (M^+) 83, 97, 111
5	23.85	C_{27}	+	+	+	+	**380** (M^+)
6	24.48		+	+	+	+	
7	24.60		+	+	+	+	
8	25.18	C_{29} monoene	+	+	+	+	**406** (M^+) 83, 97, 111
9	25.26		–	–	+	+	
10	25.33	C_{29} monoene	–	–	+	+	**406** (M^+), 83, 97, 111
11	25.45	C_{29}	+	+	+	+	**408** (M^+)
12	27.3		+	+	+	+	**434** (M^+) 83, 97, 111
13	27.36		+	+	–	–	
14	27.40		–	–	+	+	
15	27.53		–	–	+	+	
16	27.66	C_{31}	+	+	+	+	436 (M^+)

[a] Peaks are numbered in order of elution from a DB-5 capillary column and correspond with those in Figure 3.2; "+" indicates a compound is present and "–" indicates it is absent. [b] Molecular ions in bold were observed while molecular ions in normal font were not observed but could be inferred from the diagnostic ions.

Table 3.2 Univariate F statistics and P-values for the effects of *Sphenophorus* species, sex, and the interaction of these two factors on mean relative abundance of individual cuticular chemical components (df=28).

Peak #[a]	Species		Sex		Species*Sex	
	F	p	F	p	F	p
1	2.73	0.111	1.54	0.226	0.05	0.820
2	0.01	0.924	**9.79**	**0.004**	1.82	0.190
3	0.11	0.740	**10.54**	**0.003**	<0.01	0.990
4	**103.23**	**<0.001**	**13.94**	**<0.001**	**13.94**	**<0.001**
5	3.41	0.077	**8.43**	**0.008**	0.05	0.833
6	**17.87**	**<0.001**	**18.58**	**<0.001**	0.183	0.672
7	0.43	0.519	**5.46**	**0.028**	0.02	0.899
8	2.92	0.100	<0.01	1.000	<0.01	1.000
9	**341.59**	**<0.001**	**35.41**	**<0.001**	**35.41**	**<0.001**
10	**62.91**	**<0.001**	**22.00**	**<0.001**	**22.00**	**<0.001**
11	0.62	0.438	0.57	0.459	**6.25**	**0.019**
12	**30.16**	**<0.001**	0.09	0.770	<0.01	0.950
13	**47.89**	**<0.001**	2.22	0.148	2.22	0.148
14	**180.94**	**<0.001**	3.52	0.072	3.52	0.072
15	**117.88**	**<0.001**	0.27	0.609	0.27	0.609
16	<0.01	0.985	2.65	0.116	3.86	0.061

[a] Peaks correspond with those in Figure 3.2 and Table 3.1.
Significant statistical values are indicated in bold.

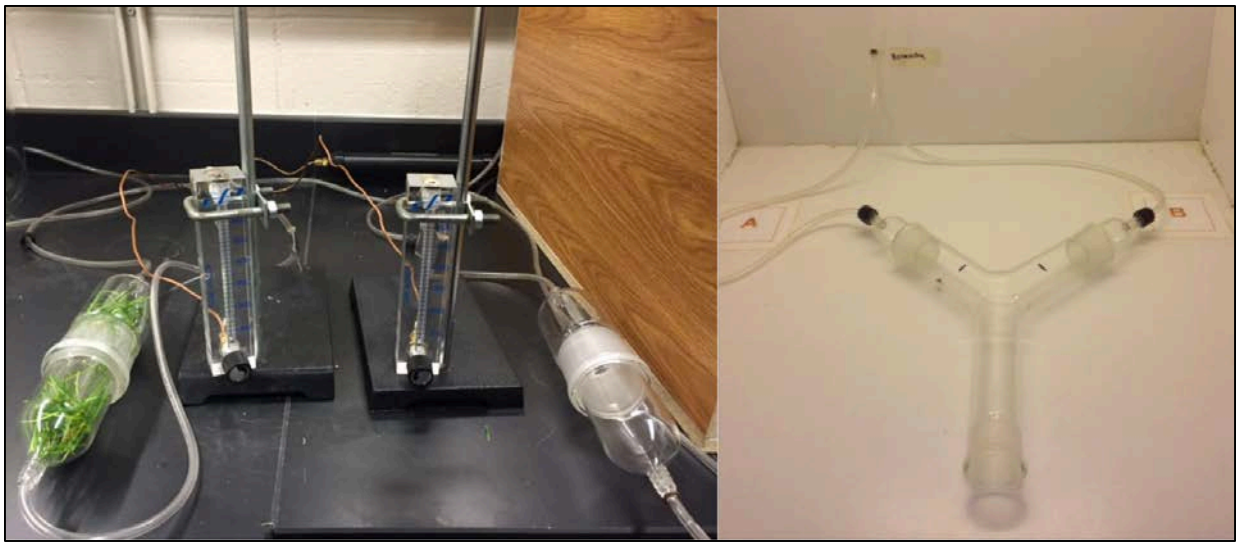
Table 3.3 Mean (± SE) relative abundances of cuticular hydrocarbon components for *Sphenophorus venatus* and *S. parvulus*.

Peak #[a]	Percent of total hydrocarbons[b]			
	S. venatus		*S. parvulus*	
	Female	Male	Female	Male
1	1.48±0.71 a	1.93±0.64 a	2.84±0.29 a	3.99±1.0 a
2	3.33±0.52 ab	5.20±0.63 ab	2.09±0.49 b	6.11±1.18 a
3	1.42±0.42 a	2.52±0.83 a	1.14±0.05 a	3.38±0.52 a
4	ND	ND	3.24±0.56 b	5.83±0.71a
5	24.10±3.56 ab	13.96±1.47 b	30.33±4.31a	19.53±5.25 ab
6	10.14±1.11 b	16.76±1.73 a	2.78±0.40 c	10.27±1.70 b
7	2.76±0.31 a	3.82±0.81 a	2.31±0.23 a	4.01±0.80 a
8	6.56±2.21 a	6.27±1.61 a	4.05±1.04 a	3.03±0.69 a
9	ND	ND	10.75±2.09 a	4.47±0.54 b
10	ND	ND	5.39±0.54 a	1.25±0.65 b
11	17.38±1.45 a	13.14±1.03 a	13.77±0.81 a	17.44±2.05 a
12	16.09±3.09 a	14.66±1.12 a	3.00±0.75 b	1.97±0.52 b
13	10.87±2.56 a	17.45±0.94 a	ND	ND
14	ND	ND	8.04±0.85 a	6.22±0.77 a
15	ND	ND	6.75±1.16 a	6.46±0.83 a
16	4.99±0.39 a	4.31±0.42 a	3.53±0.33 a	6.04±0.89 a

[a] Peaks correspond with those in Figure 3.2 and Table 3.1.

[b] Differences between mean relative abundances of *S. venatus* males (n=10) vs. females (n=10) and *S. parvulus* males (n=8) vs. females (n=5) were tested with MANOVA followed by HSD Tukey test. Means in the same row followed by the same letter are not significantly different ($\alpha<0.05$). ND=not detected.

Figure 3.1 Y-tube olfactometer used to characterize the response of *Sphenophorus venatus* beetles to Bermudagrass *C. dactylon* volatile organic compounds (VOC's) and/or conspecifics.



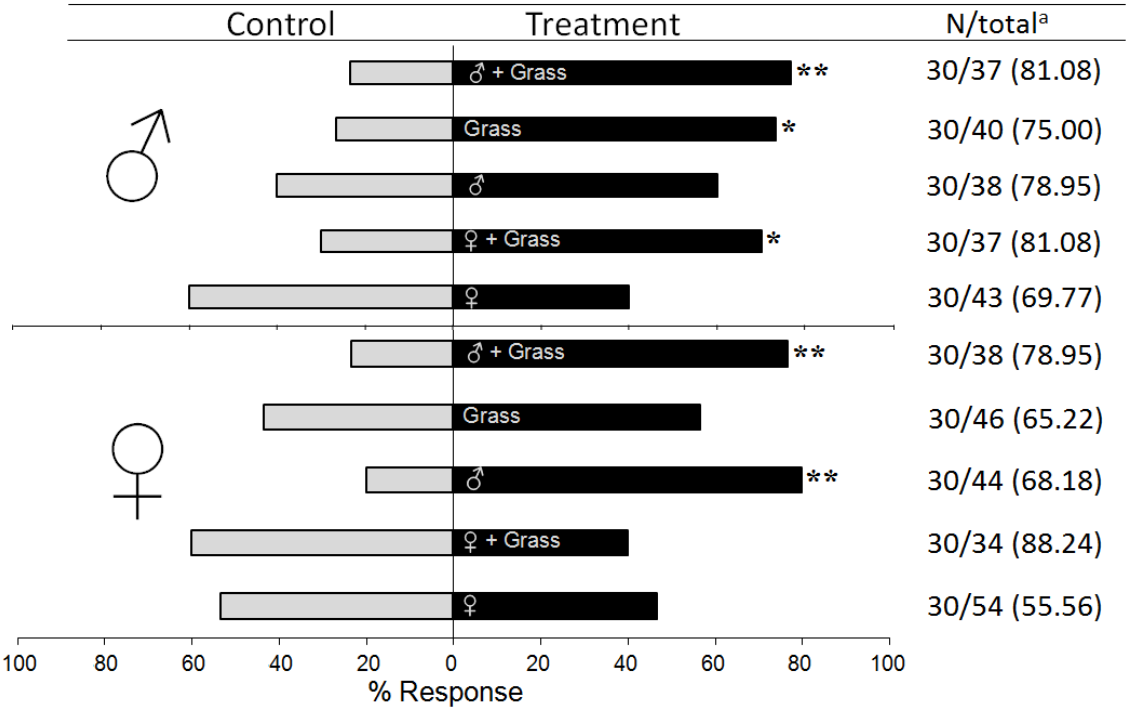


Figure 3.2 Percentage of *S. venatus* adults responding to five combinations of conspecifics and/or host-plant material vs. purified air in a y-tube olfactometer bioassay. *P<0.05, **P<0.01 (Observed vs. Expected Chi-Square). [a] The total number of billbugs responding to an odor source (N) divided by the total number of billbugs tested (percent of responsive billbugs).

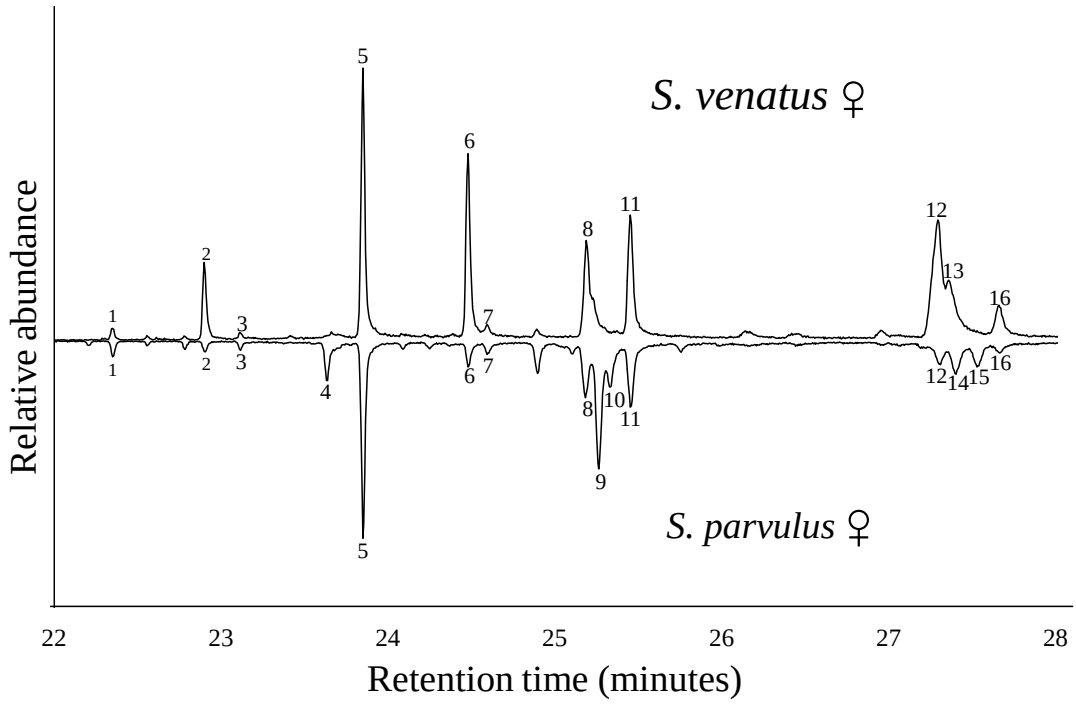


Figure 3.3 Representative total ion chromatogram of *Sphenophorus venatus* (top) and *S. parvulus* (bottom, inverted) female (♀) cuticular hexane extracts. Numbers above peaks correspond with those in Table 3.1.

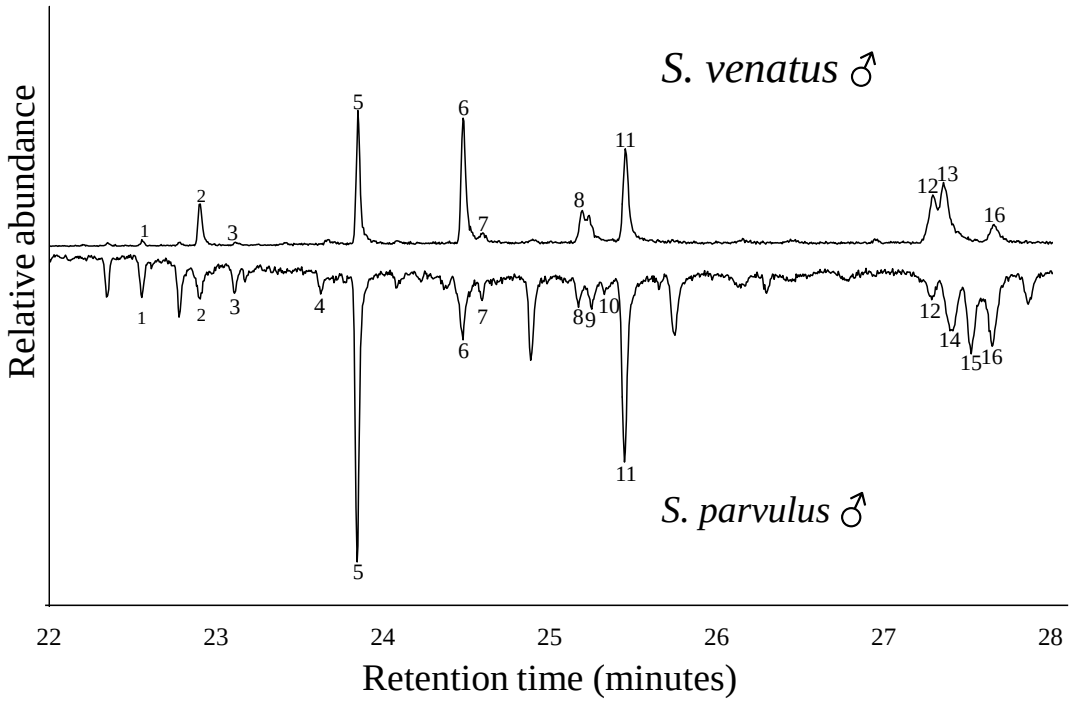


Figure 3.4 Representative total ion chromatogram of *Sphenophorus venatus* (top) and *S. parvulus* (bottom, inverted) male (♂) cuticular hexane extracts. Numbers above peaks correspond with those in Table 3.1.

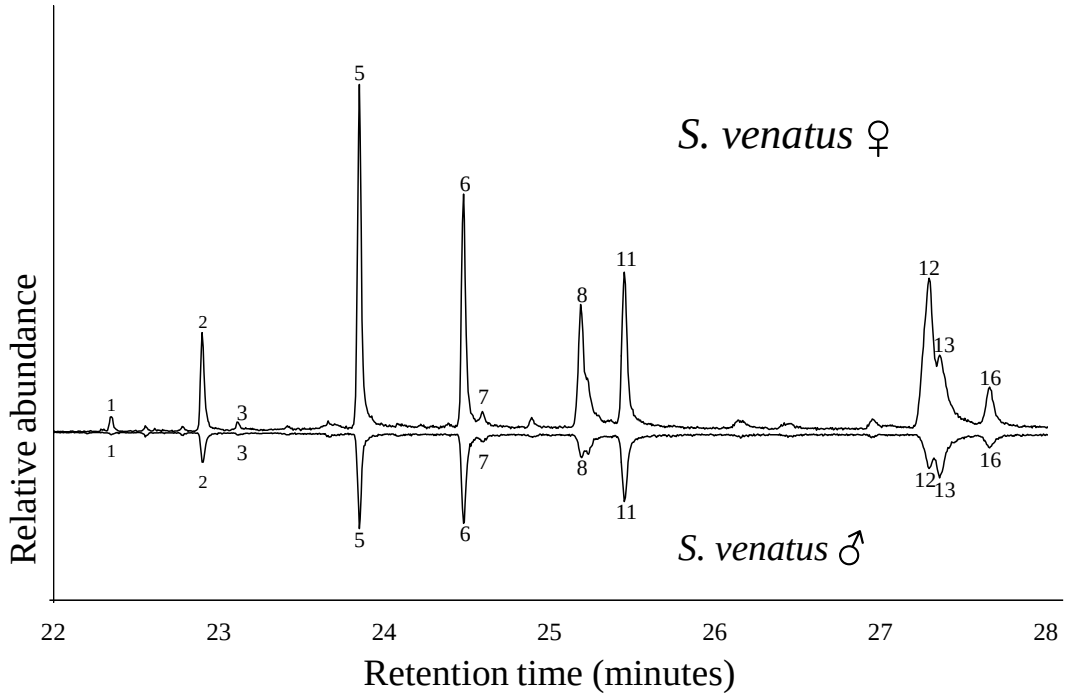


Figure 3.5 Representative total ion chromatogram of *Sphenophorus venatus* female (♀) (top) and *S. venatus* male (♂) (bottom, inverted) cuticular hexane extracts. Numbers above peaks correspond with those in Table 3.1.

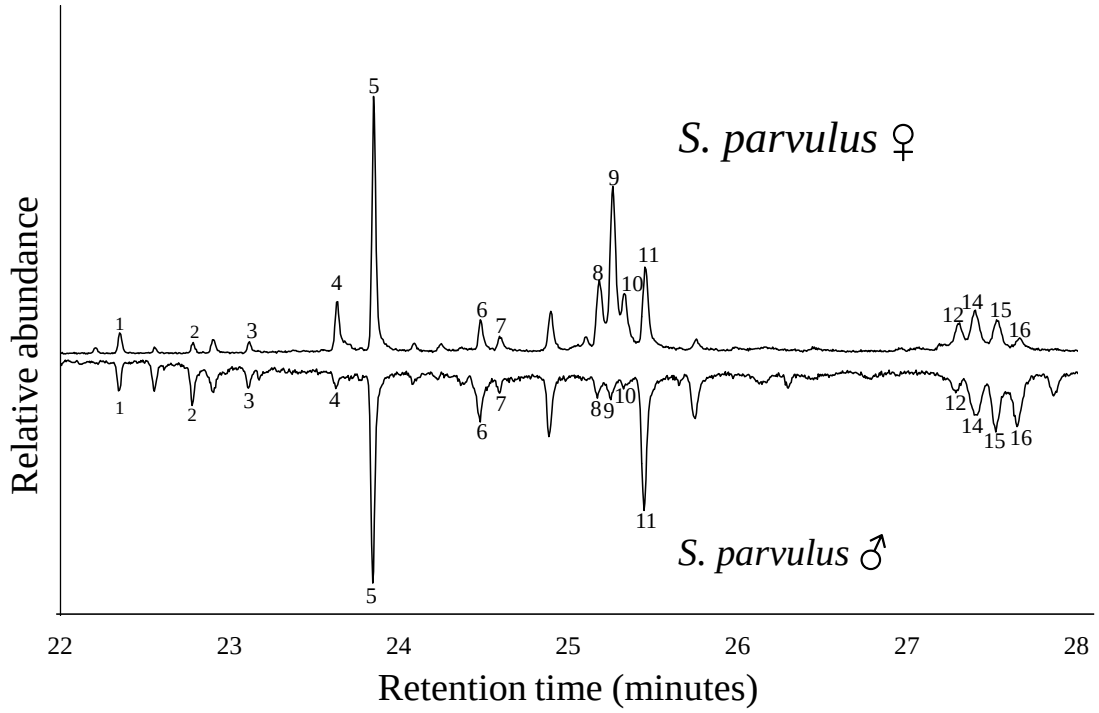


Figure 3.6 Representative total ion chromatogram of *Sphenophorus parvulus* female (♀) (top) and *S. parvulus* male (♂) (bottom, inverted) cuticular solvent extracts. Numbers above peaks correspond with those in Table 3.1.

CONCLUSIONS

The overarching goal of this research was to contribute to the understanding of billbug biology and behavior in an effort to support the formulation and improvement of integrated pest management (IPM) strategies. This thesis described the species composition of billbugs associated with managed turfgrass in Indiana and clarified *Sphenophorus venatus* seasonal biology in the Midwest. In addition, molecular techniques were used to identify sympatric, turf-inhabiting billbug species, and the potential for billbugs to employ two modes of chemoreception, 1) recognition of long-range volatile organic compounds and 2) close-range cuticular wax components, was investigated

Findings from this thesis could have immediate impacts on billbug management and provide the foundation for future research and development of sustainable IPM strategies. The occurrence of multiple sympatric species, such as the four sympatric species collected in this study in Indiana, complicates insecticide programming for turfgrass managers. In lieu of the three insecticide-based management strategies commonly adopted for billbugs in the Midwest, results of the present study underscore the necessity for turfgrass managers to monitor adult populations and be fully informed about the composition of billbug species common in their region, especially if *S. venatus* is present.

S. venatus differed in its overwintering behavior and seasonal phenology compared to the other three species that were collected. *S. venatus* adult activity is initiated earlier in the spring than the other species and, based on molecular life-stage association, is capable of overwintering in the larval stage in the Midwestern U.S. Overwintering in both the adult and larval stages resulted in two separate, overlapping cohorts that were present during the majority of the growing season. Based on its more complex seasonal phenology, insecticide-based management strategies that target both adults and larvae may prove successful for managing populations where *S. venatus* is present. Monitoring with pitfall traps is helpful to determine when adults first become active in the spring, allowing for early and accurately timed preventative insecticide applications and potentially reducing the need for subsequent insecticide inputs later in the year. Semiochemicals may prove useful for enhancing these monitoring efforts. Results from this thesis indicated that billbugs will orient to VOCs, but more research is necessary to identify the specific compounds mediating this behavior before synthetic lures can be developed and employed in IPM programs.

The occurrence of multiple sympatric species also instigated questions on billbug behavior, such how these insects find each other and how they distinguish mates. Investigation of chemically-mediated behavior in billbugs suggested that 1) *S. venatus* males are predominantly attracted to host-plant volatiles, 2) the presence of a male-produced volatile sex pheromone in *S. venatus* that is attractive to female conspecifics, and 3) qualitative and quantitative differences in the cuticular hydrocarbon profiles of *S. venatus* and *S. parvulus* could be crucial for mate recognition for sympatric *Sphenophorus* species. In addition, pitfall monitoring efforts from Chapter 2 indicated a

male-biased sex ratio throughout the growing season. These findings support the hypothesis that males are more active or mobile than females and potentially initiate colonization of host plants. Turfgrass managers should closely monitor *S. venatus* adult populations during spring-green up of warm-season grasses, as the first mowing event in the spring may initiate production of VOCs that initiate male dispersal from overwintering sites. These males likely colonize host plants and then produce a pheromone to attract females. Females likely direct their movement towards this putative male-produced pheromone, where they feed, find potential mates, and oviposit. This directed movement could explain why less females were captured in pitfall traps, which work by passively intercepting beetles as they walk across the soil surface.

Because as many as 11 sympatric billbug species infest turfgrass and preliminary *S. venatus* mating behavior observations align with those associated with weevils that use contact pheromones, the cuticular hydrocarbon patterns of *S. venatus* and *S. parvulus* were investigated. Analyses of cuticular extracts indicated qualitative and quantitative interspecific chemical differences between cuticular hydrocarbon profiles of *S. venatus* and *S. parvulus*, as well as intraspecific quantitative differences between males and females of the same species. These findings support the idea that cuticular hydrocarbons could play a role in mate recognition between closely-related, sympatric species. Although more research will be necessary to confirm the behavioral activity of these cuticular hydrocarbons, they could potentially be exploited for the development of mating disruption strategies, such as a waxy coating of cuticular components applied to fertilizer granules to confuse males and disrupt mating. In addition, these findings generate basic questions on sympatric speciation that could potentially integrate molecular methods

from Chapter 2 and analysis of cuticular hydrocarbons in Chapter 3 to investigate the evolution of chemical cues in closely-related species.

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