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Predator Defense and Host Selection Behavior of Billbugs (Coleoptera: Dryophthoridae)

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PREDATOR DEFENSE AND HOST SELECTION BEHAVIOR OF BILLBUGS

(COLEOPTERA: DRYOPHTHORIDAE)

by

Desireè M. Wickwar

A thesis submitted in partial fulfillment
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ABSTRACT

Predator Defense and Host Selection Behavior of Billbugs (Coleoptera: Dryophthoridae)

by

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Utah State University, 2021

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Billbugs (Coleoptera: Dryophthoridae: *Sphenophorus* sp.) are a complex of weevil species that damage turfgrass. These pests are traditionally managed with applications of preventive, systemic insecticides. However, management of billbugs in turfgrass poses a unique set of challenges that necessitate the development of additional, non-chemical management tools to increase efficacy and ease of billbug control, and diversify control tactics. Here I investigate billbug behavior in relation to two key components of integrated pest management programs that could reduce reliance on chemical control: biological control, leveraging resident predators to suppress billbugs (Chapter II) and cultural control, leveraging turfgrass traits to reduce pest pressure (Chapter III). In Chapter II, I investigate the effects of predators and cues associated with predator presence on billbug behavior. Billbugs responded to the presence of predators with reduced feeding and mating, and increased time spent in predator avoidance. Moreover, predator odor alone induced similar behavioral changes. These results suggest that, though predators do not often directly consume billbugs, their presence may still contribute to billbug suppression. My findings also provide the framework for further investigation of predator chemical blends as

a potential billbug management tool. In chapter III, I examine adult billbug preferences for water stressed turfgrasses, and turfgrass cultivars with different drought resistance traits. In surveys, I found that billbugs were more abundant in drier turfgrass areas, and that billbug damage was higher in Kentucky bluegrass cultivars with lower drought resistance. In choice assays I found that, while billbugs did prefer some cultivars over others, they did not prefer drought stressed or drought susceptible cultivars. Though drought resistant turfgrass cultivars are available to alleviate drought stress and may generally suffer less billbug damage, this does not appear to be a function of repelling adult billbugs. Given my observations that billbug preference and damage differ between turfgrass cultivars, continued evaluation of the factors that drive these differences could guide cultivar development and selection to maximize resistance to both drought and billbug damage, two key stressors impacting turfgrass in the Intermountain West. Overall, my research shows that short term changes in management practices, such as predator conservation and cultivar selection, could assist in billbug management. Future research could develop management strategies using predator chemicals and pinpoint turfgrass traits responsible for billbug resistance, forming the basis for integrated pest management programs for billbugs.

(136 pages)

PUBLIC ABSTRACT

Predator Defense and Host Selection Behavior of Billbugs

Desireè M. Wickwar

Billbugs are a complex of weevils that feed on the roots of turfgrass, causing severe damage to the plants. These pests are traditionally managed with applications of insecticides. However, there is a need for non-chemical management tools. Here I investigate billbug behavior in relation to two potential avenues for more sustainable management: using resident predators to suppress billbugs (Chapter II) and selecting specific turfgrasses that resist billbug damage (Chapter III). In Chapter II, I investigate the effects of predator presence and cues associated with their presence on billbug behavior. Though resident predators contribute very little to billbug suppression through directly killing and consuming billbugs, I found that the presence of predators caused billbugs to spend less time feeding and mating, and more time on predator avoidance behaviors. Moreover, predator odor alone induced similar changes, suggesting that adult billbug detect predators using their odor. Although predators do not often directly consume billbugs, their presence may still contribute to billbug suppression. My findings also provide the framework for further investigation of predator chemicals as a potential billbug management tool. In chapter III, I examine adult billbug preferences for water stressed turfgrasses, and turfgrass cultivars with different drought resistance traits. I found that billbugs were more abundant in drier areas, and that billbug damage was higher in turfgrass with low drought resistance. While billbugs did prefer some turfgrasses over others, they did not prefer drought stressed or drought susceptible plants. Drought resistant turfgrasses are available to alleviate drought stress and may generally suffer less billbug damage, however, this does not

appear to be a function of repelling adult billbugs. Continued evaluation of the factors that drive

billbug preferences among turfgrasses, and turfgrass of traits associated with lower billbug

damage could guide cultivar development against two key stressors, drought and billbugs, in the

Intermountain West. Overall, my research shows that short term changes in turfgrass

management practices, such as conserving the natural predator community and selecting specific

types of turfgrass, could assist in billbug management. In the long term, investigation of billbug

management using predator chemicals and development of turfgrasses that resist billbug damage

could form the basis of sustainable billbug management programs.

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CHAPTER I

LITERATURE REVIEW

1. The Turfgrass System and Management Challenges

In the United States (US) turfgrass covers three times as much land area as any other irrigated crop, with an estimated total coverage of more than 40 million acres (164,000 km^2), comprised of many different use types, from small lawns to large-scale turf farms and golf courses of many acres (Milesi et al., 2005). However, turf differs from other systems of a similarly large-scale, namely crops, in that it is valued primarily for its aesthetic value. Acceptable levels of damage in turfgrass are therefore vastly lower than in most large-scale crops. While premier golf courses and home owners often expect to maintain 100% green-cover, growers of crops like soybeans and cotton will accept levels of foliage loss and plant death ranging from 15-30% before even beginning to treat for pests (Crow et al., 2021). The scale of turf, coupled with these strict standards for damage, make it a difficult system to manage. Furthermore, the turfgrass industry faces numerous challenges, as many of its common management practices and tools come under increased regulation and scrutiny (Bélair et al., 2010). New regulations for pesticide use in turfgrass have been adopted throughout much of the developed world, and though the US lags behind many nations in banning pesticides (Donley, 2019), there have been some shifts toward increased regulation in the US as well (Clark and Kenna, 2000). Increased regulation of water use also may impact turfgrass, as the frequency and severity of drought events leads to more frequent irrigation bans in many regions, particularly in the western US (Cook et al., 2018; Williams et al., 2020). Despite the fact that these shifts in regulations and public expectation place limits on turfgrass management, there has only been an increase in the demand for healthy, uniform turfgrass (Bélair et al., 2010). To fulfill this demand,

while also contending with a changing management landscape, there is increasing need to find alternative ways to manage turfgrass pests and stressors to provide healthy, uniform fields, while also decreasing the need for input of chemicals and water resources (Held and Potter, 2012).

2. Billbug Distribution

Billbugs were first recognized as a serious pest in turfgrass systems in the 1960s after a series of outbreaks that were attributed to evolved pesticide resistance (Tashiro and Personius 1970). Though billbugs have been documented to use a wide variety of grass plants as hosts, including crops such as corn, wheat, rye and rice, as well as forage, and range grasses, turfgrass remains the primary focus in billbug literature (Asay et al., 1983; Kuhn et al., 2013; Satterthwait, 1931). The common name "billbug" can refer to any one of a number of weevil species in the genus *Sphenophorus,* which contains 71 species worldwide (Johnson-Cicalese et al., 1990). At least 10 of these species of billbug are known to infest turfgrasses from southern Canada, throughout the contiguous US, and into northern Mexico, as well as in Hawaii, Puerto Rico, the Bahamas, and the Dominican Republic (Kuhn et al., 2013).

Each billbug species is unique in its range and host preference. Overlap in these ranges leads to the formation of different species complexes in different regions. The bluegrass billbug (*Sphenophorus parvulus* Gyllenhal) predominates in the northern half of the US (Dupuy and Ramirez, 2016), but can be found in nearly every state in the continental US (Johnson-Cicalese and Funk, 1990; O'Brien and Wibmer, 1982; Tashiro, 1987; Tashiro and Personius, 1970). This common species tends to damage Kentucky bluegrass (*Poa pratensis* L.) and perennial ryegrass (*Loium perenne* L.) (Kuhn et al., 2013; Tashiro and Personius, 1970). Hunting billbug (*S. venatus vestitus* Chittenden), another of the most abundant and ubiquitous of billbug species, is present throughout transitional turf areas, but dominates southeastern US turf, as well as Mexico

and Hawaii, Puerto Rico, the Bahamas, and the Dominican Republic (Kuhn et al., 2013; O'Brien and Wibmer, 1982; Reynolds et al., 2016). Hunting billbugs tend to damage warm season turfgrasses such as bermudagrass (*Cynodon* spp.) and zoysiagrass (*Zoysia* spp.) (Doskocil and Brandenburg, 2013; Huang and Buss, 2009), but can also damage cool season turfgrass (Johnson-Cicalese et al., 1990; Johnson-Cicalese and Funk, 1990). Other species are present along with these two to form the characteristic species complexes in different regions. In the Rocky Mountain region and in the northern Great Plains, as well as in parts of the northwestern US, the Rocky Mountain billbug (*S. cicatristriatus* Fabraeus) is found along with bluegrass and hunting billbugs (Dupuy and Ramirez, 2016; O'Brien and Wibmer, 1982; Rondon and Walenta, 2008). However, Rocky Mountain billbug has been documented in areas outside those thought to be its primary range, such as south-central Mexico, Texas, Alberta, North Dakota and Minnesota, so it may be more widespread than originally proposed. This species tends to damage Kentucky bluegrass and perennial ryegrass (Asay et al., 1983). In the southwestern US, California and Idaho the 3-member species complex of the Great Plains also includes the Phoenician billbug (*S. phoeniciensis* Chittenden), which tends to damage bermudagrass and zoysiagrass (O'Brien and Wibmer, 1982; Sutherland, 2006). In the northeastern US the species complex includes the bluegrass and hunting billbug, as well as the lesser billbug (*S. minimus* Hart) and unequal billbug (*S. inaequalis* Say) (Johnson-Cicalese and Funk, 1990; O'Brien and Wibmer, 1982). Other turf-dwelling species are also widespread, though less research has focused on them. These species include *S. apicalis* (LeConte), *S. coesifrons* (Gyllenhal), and *S. sayi* (Gyllenhal), all documented in states varying from Alaska to Texas and into Mexico, *S. rectus* (Say) (found mostly in the Great Plains region and southern US), and the southern corn billbug (*S. callosus* Oliver) which

seems to be found predominately in the southern and southeastern US (Gireesh and Joseph, 2020; Held and Potter, 2012; O'Brien and Wibmer, 1982; Walenta et al., 2004).

3. Billbug Biology and Life History

All adult billbugs have a snout and elbowed antennae characteristic of weevils (Blatchley and Leng, 1916). Adults range in size from around 7-11 mm, and vary in color from black/grey, to red-brown (Doskocil and Brandenburg, 2013; Niemczyk, 1983; Vittum, 2020) (Fig 1-3). These discrepancies in size and color, along with species-specific rows of pits and/or furrows on their elytra and thorax, and the shape of the smooth, raised area on the pronotum are the traits primarily used to differentiate between billbug adults (Blatchley and Leng, 1916; Shetlar and Andon, 2012). Further differences exist as well, with hunting billbugs being predominantly nocturnal, while all other species are generally thought to be diurnal (Huang and Buss, 2009). Adult billbugs have wings but are essentially flightless (Young, 2002). Nevertheless, they are highly mobile within the sward. Adults are often seen "wandering" within it, and across cement and asphalt surfaces on warm days, and walking is thought to be their primary mode of travel (Kamm, 1969). Adults puncture a small hole in the stem or stolon of grasses through which to feed. In some species, these feeding holes are used as oviposition sites as well, while in other species separate slits are chewed in stems and stolons into which eggs are laid (Watschke et al., 2013). These eggs are opaque white, elongate and around 1.5 mm in length (Vittum, 2020).

The first instar larvae hatch out inside the stem of the grass, and feed within the plant. When they can no longer fit within the stem, larvae move out of the plant and into the thatch/soil layer where they begin to feed upon roots and stems of grasses, often severing roots, or cutting stems off at the crown (Johnson-Cicalese et al., 1990). Larvae are legless, with a body white-cream in color that is often larger in diameter than the head capsule, which is a red-brown color

(Satterthwait, 1931; Vittum, 2020) (Fig 4). Mature larvae, found in the thatch layer or buried a few centimeters below the soil surface, may reach sizes of 6-10 mm (Kuhn et al., 2013; Satterthwait, 1931). To date, no consistent morphological methods for differentiating between larvae of different billbug species have been identified, and researchers rely upon rearing immatures to adulthood or genetic analyses for species identification (Duffy et al., 2018b).

Pupation occurs in soil chambers at a depth of around 2.5-5 cm (Blatchley and Leng, 1916; Kuhn et al., 2013; Watschke et al., 2013). The snout of the weevil is clearly visible in the pupal stage, and the wing pads and legs can also be seen folded against the body (Satterthwait, 1931). Pupae are oblong and begin as cream-color, slightly smaller than the final instar larvae from which they arose, then slowly change to reddish brown as they near emergence.

Billbug development generally occurs between April and September through much of the northeastern and northwestern United States, and from March through October in the Intermountain West (Blatchley and Leng, 1916; Dupuy and Ramirez, 2016). In the southernmost reaches of their range, hunting billbugs may have multiple generations per year (Huang and Buss, 2009; Watschke et al., 2013; Young, 2002). Some populations of hunting and Rocky Mountain billbugs also seem to attempt a second generation even in cooler climates such as in New Jersey, northeastern Oregon, Indiana, and North Carolina were significant number have been observed overwintering as larvae (Doskocil and Brandenburg, 2013; Duffy and Richmond, 2015; Johnson-Cicalese et al., 1990; Rondon and Walenta, 2008).

In populations where only one generation per year occurs, billbugs overwinter as adults (Blatchley and Leng, 1916). The nature of overwintering sites appears to vary widely. Adults having been found overwintering buried head-first in the thatch, in worm holes, or in small crevices, as well as in the junctions between impervious surfaces (i.e. pavement or buildings) and

soil (Kindler and Spomer, 1986; Niemczyk, 1983; Richmond, 2015). Protected areas beneath hedges, piles of leaf-litter, or within heavy thatch layers in unmanaged turf areas can also be used as shelter for overwintering (Richmond, 2015; Young, 2002). Adults begin to overwinter around October and November, and become active again as soon as the soil temperature begins to rise, as early as March, and extending into mid-May (Dupuy et al., 2017; Watschke et al., 2013). Females begin to lay eggs soon after becoming active, inserting single eggs into grass stems just above the crown (Kuhn et al., 2013). A single female can lay over 200 eggs over the course of her lifetime and will continue to lay into mid-June (Reynolds et al. 2016). Once laid, eggs hatch in around 6-14 days, depending upon the billbug species and the ambient temperature (Satterthwait, 1931). After 35-55 days, the final instar larvae burrow into the soil and create the pupation chambers in which they will reside for about 8-10 days (Blatchley and Leng, 1916). New adults emerge in the late summer and early fall and subsequently overwinter as temperatures drop, beginning the cycle anew (Vittum, 2020; Watschke et al., 2013).

4. Billbug Monitoring

Billbug damage is easily misdiagnosed (Niemczyk, 1983). Billbug larvae feed upon the roots and crown of turfgrass, causing spreading patches of dead or dying turfgrass which can easily be mistaken for drought stress, dormancy, various turf diseases, or damage from other insects (Potter, 1998). Part of the difficulty in diagnosing billbugs as the cause of such damage results from the cryptic nature of the damaging phase, the soil-dwelling larvae. Larvae can only be directly monitored through destructive and labor-intensive soil sampling, and even this method is only effective for detecting late instar larvae in the soil, not early instar larvae that reside in the stem of turfgrasses (Held and Potter, 2012). By the time larvae are in the soil and can be monitored in soil cores, they are already feeding upon the roots and crowns of turf, and it

may be too late to prevent visible damage (Richmond, 2015). However, recently developed degree day models allow managers to predict when different billbug stages will be present throughout the year, despite the difficulties in directly monitoring cryptic stages (Duffy et al., 2018b; Dupuy et al., 2017). Once damage has occurred, it can also be differentiated from other forms of stress with the "tug test", performed simply by pulling upward on the aboveground tissue of the plant (Richmond et al., 2000). If the plant breaks at the crown, and stems contain frass, it is likely that damage is a result of billbug feeding.

Ground active adults can be sampled using non-destructive methods such as pitfall trapping, sweeping, or by simply noting when and where adults are observed. Though the adults are not the damaging phase they are much easier to monitor, so being able to predict larval abundance from adult abundance the previous year would be beneficial for managers. Evidence of such a correlation was found in the turf-dwelling annual bluegrass weevil (*Listronotus maculicollis*), where adult numbers one year significantly predicted subsequent local larval density and damage the following year (Mcgraw and Koppenhöfer, 2009; McGraw and Koppenhöfer, 2010). However, many environmental factors (i.e. soil moisture) influence billbug larval survival rates, so further study is necessary to determine if this pattern holds true for billbugs (Reynolds et al., 2016).

5. Integrated Pest Management

While billbug management continues to rely heavily upon synthetic insecticides, there have been developments in our understanding of billbug biology and ecology that could facilitate a more integrated approach. Integrated pest management (IPM) is a systems-based approach that draws from a diverse array of management tools to keep pest populations below thresholds where they inflict unacceptable levels of damage, while also limiting monetary, human, and

ecological costs (Williamson, 2007). IPM takes a sequential approach to pest management, with preventative measures occurring before, and hopefully precluding the need for, corrective measures (Barzman et al., 2015). The elements of IPM, in order of their suggested implementation are: 1) cultural control, 2) biological control, and 3) chemical control (Stenberg, 2017).

5.1 Cultural control

Cultural control refers to the control of pest populations or damage in a system through the alteration of that system itself or specific management practices within that system (Herzog and Funderburk, 1986). Such alterations often aim to alter host plants to minimize pest damage, or to reduce survival of pests through creating a less suitable habitat.

5.1.1 Management practices

In turfgrass, avoiding plant stress from other biotic and abiotic sources can help turfgrasses suffer less visible billbug damage (Shetlar and Andon, 2012). Promoting plant health through sufficient irrigation and fertilization can foster vigorous growth and production of new shoots, concealing damaged or dying plants (Shetlar and Andon, 2012; Watschke et al., 2013). However, the same practices that favor turf health and quality —such as regular irrigation, mowing, and fertilization— can create favorable conditions for many species of turf pests if not implemented correctly (Held and Potter, 2012). For instance, heavy fertilization can lead to the development of a thick thatch layer. If not removed, this layer then creates a refuge for billbugs, and blocks the penetration of insecticides used to manage them (Kindler and Spomer, 1986). Extremely short mowing heights also stress turf, so maintaining mowing heights on the upper end of what is acceptable for the desired turf use may also assist in reducing stress and

preventing billbug damage (Held and Potter, 2012). Overall, cultural control shows promise as a tool for billbug management. Slight alterations can be made to common management practices to provide an economically and environmentally sound strategy for management of billbugs and other turfgrass pests.

5.1.2 Plant Resistance

Thousands of cultivars have been assessed for their resistance to various pests and stressors, as well as for their general functional and aesthetic quality, with more developed each year (Bonos et al., 2006; Bushman et al., 2012; Chai and Sticklen, 1998). Though development of cultivars specifically for billbug resistance is not common, there does appear to be some genetic basis for resistance to billbugs that could form the basis for selective breeding, as turfgrass development and testing is already a well-established industry (Asay et al., 1983; Casler et al., 2003). Damage inflicted by billbugs is most apparent in turfgrass already suffering from another form of stress (i.e. drought stress), so using cultivars less prone to common stressors is one way to potentially reduce the aesthetic impact of billbug feeding (Watschke et al., 2013). Other traits, such as leaf fineness and toughness also may confer resistance to billbugs (Ahmad and Funk, 1982; Lindgren et al., 1981). Fine stems likely are less favorable as oviposition sites, as eggs laid within fine stems may face increased desiccation and predation risk as compared to eggs laid in thick-stemmed turfgrasses (Bruneau, 1987). Fine stems are also less favorable for larvae, as there is less space for the larvae to develop within the stem. This spatial restriction forces early instar larvae to drop out of the stem and into the soil, where they are at greater risk, earlier than they would with more space within the stem. Other turfgrass qualities, such as vigorous growth and rapid shoot production, may suffer less visible billbug damage simply concealing damaged or dying tissue (Johnson-Cicalese and White, 1990).

Plant resistance research targeting billbugs has largely focused on the impact of endophyte enhancement on billbug feeding and health (Johnson-Cicalese et al., 1989). Endophytes are mutualist fungi that live within certain varieties of perennial ryegrass (*Lolium perenne*) and fescue (*Festuca arundinacea*) (White et al., 1993). First, endophytes may be beneficial as a billbug management tool as they appear to generally improve the stress tolerance of turfgrasses, potentially reducing susceptibility to damage from insects such as billbugs (Johnson-Cicalese and White, 1990; Pottinger et al., 1985; Richardson et al., 2009). Furthermore, plants infected with endophytes produce several biologically active alkaloids and other toxins that provide resistance to insect feeding (Funk et al., 1993). Endophyte enhanced turfgrasses have shown resistance to many turf-dwelling insects, including billbugs, hairy chinch bugs (*Blissus leucopterus hirtus* Montandon), sod webworm (Lepidoptera: *Pyralidae*), and white grub (Coleoptera: *Scarabaeidae*) (Johnson-Cicalese and White, 1990; Murphy et al., 1993; Richmond et al., 2000). Though infection with endophytes does not appear to deter feeding or oviposition by adult billbugs directly, and adults do not appear to be able to discriminate between infected and endophyte-free varieties, feeding upon endophyte infected plants significantly increases mortality rates in adult billbugs (Murphy et al., 1993; Richmond et al., 2000). This increase in mortality was found in adults of four different species of billbug (S. *venatus*, S. *minimus*, S. *parvulus*, and S. *inaequalis*), and in both perennial ryegrass and tall fescue, promising broad management application (Johnson-Cicalese and Funk, 1990). Though it has not been demonstrated with billbug larvae directly, endophyte-infected turfgrass negatively affected soil-dwelling larvae of the related Argentine stem weevil (*Listronotus bonariensis*) (Barker et al., 1984). Difficulties in maintaining infection during seed storage and assessment of percent stand infection (ideally 35-40%) are barriers to the success of this cultural control strategy (Johnson-

Cicalese and White, 1990; Richmond et al., 2000; Watschke et al., 2013). However, with high

rates of cultivar development and rigorous testing, cultivar selection for endophyte enhanced

turfgrasses, as well as other high-performing cultivars, shows promise as a tool for billbug IPM.

5.1.3 Turfgrass drought resistance, stress, and billbugs management

Turf managers report that the most severe billbug damage occurs in areas under drought-

stress, and there is evidence that drought stress intensifies pest outbreaks in other systems

(Cammell and Knight, 1992; Mattson and Haack, 1987). However, we are unaware of any

studies to date directly examining the response of billbugs to drought or water stress in turfgrass.

Turfgrass plants have multiple mechanisms to survive under water limited conditions, avoiding

dormancy and death (Bonos and Murphy, 1999). The ability to survive drought can result from

drought tolerance (maintaining function despite low tissue water potential), or drought resistance

(maintaining a high tissue water potential despite limited water) (Richardson et al., 2009). Many

drought resistance and tolerance strategies, and physiological changes in turfgrass under drought

stress, may also influence suitability of that plant as a billbug host, and its susceptibility to

billbug damage.

Plants maintain ability to take up water, even in water limited conditions, in a number of

ways that may also influence billbugs. One such mechanism is to accumulate various solutes

(Jiang and Huang, 2001). Accumulated solutes may include carbohydrates and essential amino

acids, potentially making plants more nutrient dense for plant-feeding insects such as billbugs

(Brodbeck and Strong, 1987; DaCosta and Huang, 2006; Shen et al., 1989). Plants may also

increase water uptake through altering their root system, often increasing overall root volume

and/or depth (Bonos and Murphy, 1999; Marcum et al., 1995; Richardson et al., 2008). As

billbug larvae feed on turfgrass roots, and have very limited mobility and ability to seek resource

rich areas, these changes may provide more accessible plant material for feeding. However, turfgrasses under drought stress may only increase their root volume at soil depths greater than 20cm, with reduction in shallow root mass where billbugs feed (in the upper 2-8cm of soil), so the influence of drought-related root alteration is uncertain (DaCosta et al., 2004; Vittum, 2020).

Avoidance of water loss is another mechanism for plant drought resistance that may impact billbugs. Water loss via evaporation can be avoided through the thickening of the plant's waxy cuticle, as well as decreasing leaf surface area (Kopp and Jiang, 2015). For instance, in tall fescue, thicker epicuticular wax leads to maintenance of higher quality under drought stress, and thicker leaves (lower surface area to volume ratio) conferred drought resistance (Fu and Huang, 2004). These same traits that confer drought resistance may hinder billbug feeding, as thicker epicuticular wax and thicker leaves make tissue tougher and more difficult to consume for chewing insects (Raupp, 1985; Saska et al., 2020).

Water stress induces physiological and morphological changes that may also impact billbugs. Plants under drought stress may accumulate phytohormones, transcription factors, kinase cascades, and/or reactive oxygen species, many of the same compounds that plants use to aid in defense against biotic stressors (Ramegowda et al., 2020; Rejeb et al., 2014). Thus, drought stressed turf may have superior defenses to billbug feeding. Morphological changes in drought stressed turf may also reduce host favorability for billbugs. Drought stress was shown to reduce turfgrass shoot growth by over 40%, potentially providing less cover for surface-dwelling adults (DaCosta et al., 2004). However, drought stressed plants often have higher canopy temperatures, as demonstrated in both perennial ryegrass and Kentucky bluegrass (Bonos and Murphy, 1999; Jiang et al., 2009). These warmer temperatures may favor insect development, so in this regard drought-stressed plants could favor billbug outbreaks (Mattson and Haack, 1987).

Host attractiveness, and insect growth and development have been shown to be both increased and decreased by host drought stress in a number of insect species, so species-specific studies are warranted (Chidawanyika et al., 2014; English-Loeb et al., 1997; Gutbrodt et al., 2011; Huberty and Denno, 2004; Preszler and Price, 1995; Showler and Castro, 2010; Showler and Moran, 2003). Developing a greater understanding of the interaction between drought tolerance/resistance and billbug tolerance/resistance could aid in the development of cultivars best suited to both of these important stressors of turfgrass. The development and use of turf varieties and cultivars that can better withstand limited water availability and pests without the need for excessive irrigation and pesticide use will benefit changing turf management practices and demands on turfgrass systems and managers (Held and Potter, 2012).

5.2 Biological Control

The use of live natural enemies to control pests is referred to as biological control (Barzman et al., 2015; Eilenberg et al., 2001). The biological control of billbugs has consisted primarily of applications of microbial agents, and the conservation of generalist predators.

5.2.1 Microbial agents and derivatives

Pathogenic microbes, including entomopathogenic nematodes, bacteria, fungi, and viruses, all show promise as tools for turfgrass pest management when used independently and with other IPM tactics (Bélair et al., 2010; Koppenhöfer and Kaya, 1998; Zimmerman and Cranshaw, 1990). Entomopathogenic nematodes (namely *S. carpocapsae* and *H. bacteriophora*) have been shown to control billbug adults and larvae (Bélair et al., 2010). In fact, some studies have shown nematodes to have greater efficacy than the most commonly used insecticides, such as imidacloprid (84% vs. 69% control respectively) (Kinoshita and Yamanaka, 1998; Smith, 1994). Furthermore, there is evidence from studies of other turfgrass pests (white grubs,

Cyclocephala hirta LeConte and *C. pasadenae* Casey, and black cutworm, *Agrotis ipsilon*), of the compatibility of some species of nematode with synthetic insecticides, potentially providing excellent pest control (Ebssa and Koppenhöfer, 2011; Koppenhöfer and Kaya, 1998).

Microbial derivatives (i.e. macrocyclic lactones or spinosad) are often referred to as biological insecticides (Bélair et al., 2010). These compounds have been shown to have significant effects on billbug mortality (Vittum et al., 2007). Though there is a lack of field evidence showing their efficacy in billbug population control, they have proven effective in the field for other turfgrass pests (cutworms, sod webworms, and fall armyworms) (Gosselin et al., 2009).

5.2.2 Predators and parasitoids

Turfgrass contains a diverse array of resident predators. This community of predatory arthropods has been well documented and studied (Arnold and Potter, 1987; Cockfield and Potter, 1985, 1984; Dobbs and Potter, 2014; Kunkel et al., 1999; López and Potter, 2000; Terry et al., 1993; Zenger and Gibb, 2001), and includes multiple species of predatory ground beetles (Coleoptera: *Carabidae*), rove beetles (Coleoptera: *Staphylinidae*), ants (Hymenoptera: *Formicidae*), and spiders (Arachnida: *Araneae*) (Bixby-Brosi and Potter, 2012; Dupuy and Ramirez, 2019; Hong et al., 2011). Though many studies show these predators to be negatively impacted by conventional chemical treatments in turf (Dobbs and Potter, 2014), they often still persist in high abundance (Dupuy and Ramirez, 2019). These resident generalist predators have been shown to effectively consume other turfgrass pests, like the black cutworm (*Agrotis ipsilon*) (Frank and Shrewsbury, 2004; Hong et al., 2011). However, recent work shows that these predators pose very little direct consumptive risk to billbugs, so these common generalists show little promise for billbug control through direct consumption alone (Dupuy and Ramirez, 2019).

Birds, toads, and small mammals are also thought to feed upon billbugs (Young, 2002).

However, these larger predators are not suitable for use in biological control and are considered

secondary turf pests as their foraging for larvae damages turf. Though there are some known

parasitoids of billbugs, including *Zavipio (Vipio) belfragei* (Cresson) (Hymenoptera:

Braconidae) and Anaphes (*Anaphoidea*) *calendrae* (Gahan) (Hymenoptera: *Myrmaridae*)

(Satterthwait, 1931), these too have remained largely unexplored as a tool for biological control

of billbugs.

5.2.3 Non-Consumptive Effects of Predator Presence

Though most biological control efforts focus on reductions in pest abundance resulting

from consumption by predators, the mere presence of natural enemies may also aid in pest

management. Detection of predators can result in prey altering their physiology, life history,

morphology, and behavior (Benard, 2004; Sheriff et al., 2020; Sih, 1986; Thaler et al., 2012).

These alterations in flexible prey traits in response to perception of risk are referred to as risk-

induced trait responses (Peacor et al., 2020). Alterations in prey physiology and life history may

include changes in development times to reduce predation risk. For instance, *Manduca sexta*

larvae exposed to predators reduced time spent in the developmental stages most vulnerable to

predation (Thaler et al., 2012). This same strategy was observed in mayflies (*Baetis bicaudatus*

(Baetidae)), where threatened individuals matured at smaller size than those under no predation

risk (Peckarsky et al., 1993). Morphological changes have also been documented in insects. For

instance, aphid wing induction (which facilitates dispersal) has been shown to be influenced by

the threat of predation (Kunert and Weisser, 2003). Finally, risk-induced behavioral changes

documented in insects often include reductions in feeding and mating, and increased vigilance

(Lima and Dill, 1990; Peckarsky et al., 1993; Preisser et al., 2005; Werner and Peacor, 2003;

Williams and Wise, 2003). Predator avoidance, such as altered microhabitat usage and reduced movement, may also result from predator presence and limit the ability of prey to forage and feed (Peckarsky et al., 1993; Sih, 1986). Though no physiological or morphological alterations have been observed in billbugs to date, they have shown behavioral alterations such as reduced feeding and mating in the presence of predators (Dupuy and Ramirez, 2019).

The cumulative fitness costs and reduced abundance that occur as a result of risk-induced trait responses are referred to as the non-consumptive effects (NCEs) of predator presence. NCEs of predator presence can be equal or greater in magnitude to consumptive effects (Arnold and Potter, 1987; Preisser et al., 2005; Relyea, 2001; Thaler and Griffin, 2008; Werner and Peacor, 2003). Indirect effects of the trait response of one species on other species in a community, referred to as trait-mediated indirect effects, can cascade through communities (Peacor et al., 2020; Steffan and Snyder, 2010; Trussell et al., 2003). NCEs can manifest at the individual, population and community level. Individuals may suffer physiologically, with reduced growth and development (Benard, 2004), and reduced overall fitness (Peckarsky et al., 1993). Interestingly, the costs of behavioral responses to predator presence (i.e. reduced feeding) have been shown to be even greater than the costs of physiological responses (Buchanan et al., 2017). Responses to predator presence can sometimes lead to significant increases in mortality in potential prey (Stoks, 2001). For instance, in dragonfly larvae (*Leucorrhinia intacta*) larvae exposed to predator cues had higher levels of mortality and metamorphic failure than those reared in the absence of predator cues (McCauley et al., 2011). These changes in individual fitness can scale up to reduce growth at the population level (Harvell, 1992), and reduce the negative impacts of insect pests on their plant hosts (Hermann and Thaler, 2018; Steffan and Snyder, 2010).

Many different cue types can elicit risk-induced traits responses and NCEs. Chemical cues, both volatile (Hermann and Thaler, 2014) and cuticular (Takahashi and Gassa, 1995) are used by insects to detect and respond to presence of threats. Though chemical detection of predators has not been demonstrated in billbugs, they have been shown to use contact and volatile compounds to detect conspecifics (Barrett et al., 2018; Duffy et al., 2018a; Illescas-Riquelme et al., 2016). Visual and vibrational cues (Gish, 2021), as well as auditory cues (Yager, 2012) also induce trait responses. Billbugs have been shown to respond to tactile cues by "playing dead" (thanatosis) (Kindler and Spomer, 1986); but, to our knowledge, their response to visual and vibrational cues has not yet been documented. Determining what modality billbugs use to sense predators is foundational in determining prey response, and resulting suppressive effects, so studies on billbug predator detection could help form the basis of novel biological control programs leveraging NCEs (Weissburg et al., 2014).

The creation of such programs will also necessitate a greater understanding of the environmental context in which billbugs exist, as this context influences which cues are used and how they respond (Buchanan et al., 2017). Prey responses are influenced by factors as varied as host plant traits (Kersch-Becker and Thaler, 2015), the presence of heterospecifics (Ingerslew and Finke, 2018), and local community structure (Schmitz, 1998; Steffan and Snyder, 2010). The communities that billbugs are a part of and the predator communities they are exposed to differ based on the localized management and environmental traits (i.e. predator refuge availability), so predicting the influence of these factors on billbugs is difficult (Dupuy and Ramirez, 2019, 2016). Furthermore, often insects respond to more general cues and their environmental context or "landscape of fear" (Matassa and Trussell, 2011). Though the turfgrass system differs widely based on management and use type it has some common traits thought to influence prey

responses to predator presence. For instance, dense foliage limits billbugs ability to visually detect predators, and anthropogenic noise may limit their capacity for auditory and vibrational detection (Smee and Weissburg, 2006; Weissburg et al., 2014). However, further study is necessary to determine the importance of those traits, as well as other key factors such as billbugs' primary sensory modality for predator detection, and the nature and character of predator cues to which billbugs respond (Weissburg et al., 2014).

NCEs show potential to have strong, wide-ranging effects on pest populations, and preliminary research suggests that billbugs do alter their behavior in the presence of predators (Dupuy and Ramirez, 2019). Importantly for billbug management due to low consumptive risk, neither antipredator responses nor NCEs appear to always be directly linked to predation risk (Creel et al., 2017; Kunert and Weisser, 2003; Pessarrodona et al., 2019; Trussell et al., 2011). Even insects invulnerable to predation, as billbug adults appear to be (Dupuy and Ramirez, 2019), alter their behavior in the presence of predators in ways that may reduce host plant damage (Hermann and Thaler, 2018). Indirect effects of predators can also aid in biological control of pests through making them more susceptible to other predators or pathogens, potentially aiding in control efforts through that synergy (Ramirez and Snyder, 2009). However, prey appear to have ways of avoiding adverse, long-term consequences of risk-induced trait responses (Preisser and Bolnick, 2008; Thaler et al., 2012), so further investigation is needed to determine to what extent NCEs could contribute to billbug suppression in turfgrass.

5.3 Chemical Control

Billbug management currently relies heavily upon either contact insecticides, such as pyrethroids, or preventive applications of long-residual, systemic insecticides, such as neonicotinoids and anthranilic diamides (Dupuy and Ramirez, 2016). Preventive application of

contact insecticides occur in the early-spring (Held and Potter, 2012). These sprays target emerging adults, as eggs and small instar larvae are protected within the stem, and larger larvae quickly delve too deep into the soil for insecticides to be effective. The brevity of the susceptible life-stage of billbugs and of the activity of such insecticides (7-10 days) means that timing of application is particularly important for effective control using this method (Richmond, 2015). Preventive applications of longer-residual insecticides target early-instar larvae. Though these insecticides have longer activity times, increasing the chances of success for a single application of pesticide, such applications can be expensive and often a single application is insufficient to reduce abundance to desired levels (Dupuy and Ramirez 2016). Pesticides can represent a significant cost to turf managers and homeowners alike. On average 31% and 19% of lawn care companies and golf courses' total annual expenditures come from pesticides, respectively, and 20% of home-owners in the US also reported treating their lawns with pesticides (Held and Potter, 2012). In fact, overall expenditure on chemicals and other lawn and garden products amounted to over $35 billion in 2007 (Williamson, 2007).

Degree-day models predicting percent adult emergence are a tool that managers could use to increase specificity of timing for pesticide applications, and thus increase their efficacy and reduce costs. Models now exist for multiple geographic regions, and their ability to increase efficacy of pesticide applications has been experimentally validated (Duffy et al., 2018b; Dupuy et al., 2017). Despite introduction of such new management tools, billbug management still consists primarily of prophylactic applications of insecticides more based on budget limitations, and the judgement and past experience of the manager than on such models or specific action thresholds (Held and Potter, 2012).

6. Research Objectives

Management options for billbugs are extremely limited, despite the fact that they impact turfgrass throughout the US. Currently, management relies almost exclusively on preventive applications of synthetic insecticides. Though there is both a need and demand for sustainable alternatives, the development of such alternatives necessitates a more detailed understanding of billbug biology and ecology than we currently possess. Fortunately, there have been recent advancements in our understanding of billbugs' interaction with predators, and host selection tendencies that may facilitate the development of both biological and cultural control methods. Here I examine billbug behaviors that could contribute to development of such methods, with a particular focus on topics uniquely relevant in the western United States, a region that has been underrepresented in billbug research in the past. Here I aim to:

1. characterize bluegrass billbug behavior in the presence of predators and their cues to better understand the non-consumptive effects of predator presence, and the role that different predator cues may play in inducing billbug behavioral responses (see Chapter II; formatted according to the journal Biological Control guidelines)

2. examine billbug abundance in relation to soil moisture, and billbug damage in Kentucky bluegrass with varying levels of drought susceptibility. I also examine billbug host preference for Kentucky bluegrasses with varying levels of water-limitation and drought-susceptibility (see Chapter III; formatted according to the Journal of Economic Entomology guidelines)

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Fig. 1 Bluegrass billbug (*S. parvulus*) adult, dorsal (upper) and lateral (lower) views. Identifying features include even dimpling on the thorax, lacking any smooth raised areas. Bluegrass billbugs are the smallest of the billbug species common in the western United States, ranging in size from 5-7 mm. Scale bar 6.70mm (upper) and 6.75mm (lower). Photo credits: Desireè Wickwar, Utah State University.

Fig. 2 Hunting billbug (*S. venatus vestitus*) adult, dorsal (upper) and lateral (lower) views.

Identifying features include red-brown coloration, and a raised "(Y)" shaped area on the thorax.

Hunting billbugs range in size from 7-10 mm. Scale bar 10.15mm (upper) and 9.59mm (lower).

Photo credits: Desireè Wickwar, Utah State University.

Fig. 3 Rocky Mountain billbug (*S. cicatristriatus*) adult, dorsal (upper) and lateral (lower) views. Identifying features include dark black coloration, characteristic hoof-shaped indentations on elytra, and small, even dimples on covering the thorax. Rocky Mountain billbugs are the largest of the species common in the western United States, ranging in size from 10-12 mm. Scale bar 10.36 mm (upper) and 10.55 mm (lower). Photo credits: Desireè Wickwar, Utah State University.

Fig. 4 A late-instar billbug larva in turfgrass roots. Billbug larvae range in size from 6-10mm in later instars. Photo credit: Desireè Wickwar, Utah State University.

CHAPTER II

NON-CONSUMPTIVE EFFECTS OF DIFFERENT PREDATORS AND CUES ASSOCIATED
WITH THEIR PRESENCE ON BILLBUG BEHAVIOR (COLEOPTERA:
DRYOPHTHORIDAE)

Abstract

Billbugs (*Sphenophorus* sp.), a complex of weevils that damage turfgrass, are
traditionally managed with preventive, systemic insecticides. Though little direct consumption
has been described for the diverse predator community in the turf system, the presence of
predators alone may still contribute to billbug suppression by causing billbugs to alter their
behavior, and inducing suppression through non-consumptive effects. Here, we used two
different experiments to quantify the extent to which exposure to predators and predator cues
altered billbug behavior (predator avoidance, feeding and mating). First, a series of behavioral
assays conducted in microcosms exposed billbugs to common predators (*Pterostichus
melanarius, Harpalus* sp., *Philonthus* sp., and *Lycosid* sp.) singly and in combination to measure
the effect of contact with predators and potential synergy among predator species pairs. Indirect
exposure to predators, predator odor, and a non-predatory arthropod were used to measure
effects of different predator cues on billbug behaviors. In subsequent Y-tube choice assays we
determined if billbugs discriminated between filtered air and predator odor to isolate chemical
signaling as a potential mechanism for billbug predator detection. Direct exposure to predators
increased predator avoidance (up to 201%), and decreased billbug feeding (up to -78%) and
oviposition, though no predation was observed. Similar behavioral changes were observed when
billbugs were exposed indirectly to a predator, and to predator odor. Along with Y-tube assays

showing billbugs avoided treatments with predator odor, these results suggest billbugs use chemical cues to detect predator presence and drive behavior. Our results suggest that conservation of predatory arthropod communities in turfgrass could play an important role in biocontrol, even in the absence of strong direct consumptive effects, and provide the foundation for future research concerning chemical signaling.

Keywords biological control, non-consumptive effects, Y-tube, turfgrass

1. Introduction

Prey often respond to predator presence by altering their physiology, morphology, life history and behavior (Sheriff et al., 2020). These responses, collectively known as risk-induced trait responses, may have a positive impact upon prey fitness by decreasing mortality from predation; but there may also be tradeoffs in fitness costs. For instance, by increasing vigilance and time spent in defensive behaviors, prey species may reduce their risk of predation, but at the expense of decreased time spent feeding and mating, behaviors that bolster fitness (Peckarsky et al., 1993; Preisser et al., 2005; Thaler and Griffin, 2008). The effects of such behavioral changes can scale up to influence distribution of prey at a landscape level and have community-wide effects (Matassa and Trussell, 2011). The cumulative fitness cost of these risk-induced trait responses, is known as the non-consumptive effect (NCE) of predator presence (Peacor et al., 2020). In many cases the NCE of predator presence on the threatened individual has been shown to equal or exceed the consumptive effect of predators (Křivan and Schmitz, 2004; Peacor and Werner, 2001; Preisser et al., 2005; Thaler and Griffin, 2008; Trussell et al., 2006). NCEs can also extend to the population level and influence multitrophic interactions, potentially impacting not only the individual but entire populations or food webs through cascading effects (Preisser et al., 2005; Trussell et al., 2003). Due to the potential magnitude and scope of effects on pest populations, NCEs may be an underappreciated element of biological control and promising tool for integrated pest management.

In order to foster the NCEs of predator presence that may lead to pest suppression it is essential to understand what influences prey perception of risk. Cues inducing prey perception of risk may be chemical, with both nonvolatile (cuticular hydrocarbons) and volatile organic compounds (VOCs) being used by prey to detect predator presence (Hermann and Thaler, 2014).

Prey can also use tactile and visual cues to sense predator presence (Hermann and Landis, 2017; Williams and Wise, 2003), as well distinguish vibrational cues from predators versus non-predators (Gish, 2021). The traits of the predator community (i.e. species richness or diversity), and the individual predators (i.e. the risk of consumption a predator poses or its hunger state) may also influence type and magnitude of prey behavioral responses (Wirsing et al., 2021). While some studies suggest that the identity of the predator species present most strongly influences the prey's behavioral response (Murie and Bourdeau, 2019; Smee and Weissburg, 2006), others suggest that predator diversity, and nature of the predator community at large is of greater importance (Byrnes et al., 2005; Steffan and Snyder, 2010). The degree to which the consumptive threat posed by a predator influences prey response is also inconsistent across systems, varying with the mechanism used by prey to detect both known and novel predators (i.e. species-specific identification versus generalized response to disturbance cues). While some prey species respond more strongly to more lethal predators (Hill and Weissburg, 2013; Thaler et al., 2012) others do not modulate their response based on the magnitude of the consumptive threat (Creel et al., 2017; Kunert and Weisser, 2003; Pessarrodona et al., 2019; Relyea, 2001). Determining if billbug responses to predators are general or specific, and what drives behavioral responses will facilitate a greater understanding of the mechanisms they use to detect predator presence and modulate behavioral responses.

In this study we examine the predator cues that influence risk-induced behavioral responses in bluegrass billbugs (*Sphenophorus parvulus*), a pest common in the Northern United States and into Canada, where it primarily impacts cool season turfgrasses (Satterthwait, 1931; Tashiro and Personius, 1970). The feeding of billbug larvae on the roots and crowns of the plant can cause serious damage to or death in large swaths of turfgrass. Due to the cryptic nature of the soil-

dwelling damaging stage, billbugs can be difficult to detect and are one of the most frequently misdiagnosed turfgrass maladies (Niemczyk, 1983). This has led to a heavy reliance upon prophylactic applications of long-residual, systemic insecticides among managers. Though a diverse predator community can be found in turfgrass throughout North America, largely comprised of ground beetles (Coleoptera: Carabidae) such as *Pterostichus* spp., *Anisodactylus* sp., rove beetles (Coleoptera: Staphylinidae), ants (Hymenoptera: Formicidae) and spiders (Arachinida: Araneae) (Bixby-Brosi and Potter, 2012; Cockfield and Potter, 1984; Dobbs and Potter, 2014; Dupuy and Ramirez, 2019) very low rates of direct consumption (<6%) of adult billbugs by these predators have been documented (Dupuy and Ramirez, 2019).

This is the first study that attempts to isolate the cues used in billbug perception of predation risk and determine their contributions to risk induced trait responses. We are unaware of any studies that have examined this framework with bluegrass billbugs or their cues in relation to predator detection and NCEs, though studies do indicate that hunting billbugs (S. *venatus*) make use of VOCs to orient towards conspecifics (Barrett et al., 2018; Duffy et al., 2018), and billbugs often respond to tactile cues (e.g. being touched or lifted with forceps) by feigning death (thanatosis) (Kindler and Spomer, 1986). Our objective was to characterize the behavioral responses of billbug adults to the presence of predators and examine their responses to isolated predator cues (i.e. predator odor) to determine if these are used for predator detection by billbugs. To do so we used a series of microcosm assays in which billbugs were exposed to predators directly and indirectly (separated by a screen), as well as to predator odor (chemical cues) and a non-threatening arthropod, and documented behavior (feeding, predator avoidance, and mating). We also conducted a Y-tube experiment to measure billbug response to predator VOCs. An additional objective we addressed using the microcosm and Y-tube experiments was

to determine if different common predators (*Pterostichus melanarius, Harpalus* sp., *Philonthus* sp., and *Lycosid* sp.) influence billbug behavior differently, and determine the effect of single predator species versus predator species pairs.

2. Materials and Methods

2.1. Insect Collection

Adult billbugs and predators used in assays were field collected from turf areas using linear and cup pitfall traps (as described in Dupuy and Ramirez 2016). Collection sites were located throughout northern Utah including Logan Country Club, and Utah State University's Greenville Research Farm, and greenhouses (41.7661, -111.8107) and at three municipal golf courses located near Salt Lake City, Utah: Rose Park (40.8006, -111.9310), Nibley (40.7116, -111.8750), and Forest Dale (40.7185, -111.8632).

Billbugs were sorted to species and stored in containers with moistened cotton wicks at 6°C for no more than one month after field collection before use in assays. Some of the most abundant and ubiquitous predators in Intermountain West turfgrass, *Pterostichus melanarius*, *Harpalus* sp., *Philonthus* sp., and *Lycosid* sp. as described by Dupuy and Ramirez 2019, were sorted from traps, stored singly in 7-dram vials with a moistened cotton wick, and starved at 6 °C for 2–3 weeks before use in lab assays. Finally, two non-predatory arthropods that naturally cooccur with billbugs (Armadillidae and Acrididae) were collected from pitfall traps, and were stored similarly to predators. Isopods were selected as they were the most common non-predator bycatch in our pitfall traps. The grasshoppers were chosen for their abundance at our field sites, as well as for the size and high level of activity, more closely mirroring that of some of the

predators of study than did the isopod. Furthermore, two arthropod species were used to reduce the chance of documenting billbug response unique to a single non-predatory species, a possibility suggested in pilot studies (Appendix I, Fig. 1A). All arthropods used in behavioral assays were removed from refrigeration two hours before use and allowed to acclimate to room temperature.

2.2. Billbug behavior when exposed to predators and predator cues in microcosms

We used a series of microcosm assays to quantify billbug behavior when exposed to predators and predator pairings, as well as to predator cues to isolate billbug predator detection mechanisms. Microcosms consisted of a two-chamber arena (Mosquito breeder, Bioquip LLC, Rancho Dominguez, CA) (Fig. 1), the experimental unit. The lower chamber was separated from the upper chamber with a fine mesh screen (9cm from the soil surface), allowing potential indirect cues to pass between the two chambers (e.g. chemicals from predators in the indirect contact condition) (similar to Hermann and Thaler 2018). In all treatments, four adult bluegrass billbugs were placed in the bottom chamber which was filled with field soil to a depth of 2cm planted with five Kentucky bluegrass stems.

2.2.1 Treatments

To evaluate the influence of predator presence on billbug behavior we exposed billbugs to direct contact with predators by placing both billbugs and predators in the lower chamber of the arena. Treatments consisted of a control (no predator; 8 reps), 1 predator species (for each of 4 species; 4 reps/species), or 2 predator species (each combination of 2 species; 4 reps/species pairing) exposed to billbugs in the lower chamber of the arena (Fig. 1). Indirect predator treatments consisted of the treatments previously described but with predators placed in the

upper chamber of the arena, separated from billbugs in the lower chamber by a screen divider. In addition, two positive controls were set up to isolate the effect of disturbance and chemical cues arising from indirect predator contact. One positive control consisted of non-predatory arthropod in the upper chamber (for each of 2 species; 4 reps/species) as a control for disturbance cues in the absence of direct contact between billbugs and another arthropod. The other positive control of predator odor (for each of 4 species; 4 reps/species) treatment both in the upper chamber.

A substitutive design was used for treatments such that 2 predator or two non-predatory arthropod individuals were present in all treatments. The non-predatory arthropod treatments consisted of only a single species (one or the other species), but for the predator treatments the number of predator species was either 1 or 2. Each predator species (*Pterostichus melanarius*, *Harpalus* sp., *Philonthus* sp., and *Lycosid* sp.) was tested for single predator species treatments, and in all combinations for predator pair treatments. For the non-predatory arthropod treatment (positive control for disturbance cues present in the indirect predator contact treatment) two different species were used (*Armadillidae* sp. and *Acrididae* sp.) to provide a wide array of behaviors and cues (4 reps/species). The predator scent treatment (positive control for odor cues present in the indirect predator contact treatment) consisted of odor from each of the four predator species alone (n=16). Predator odor was imbued into cotton swabs by placing them in containers housing eight predators of a single species for the 72 hours prior to the assay. For the assay two cotton swabs with single predator species' odor were placed in the upper chamber of the arena.

2.2.2 Quantifying Behavior

Billbug behaviors were classified in three broad categories or global behaviors (predator avoidance, feeding, mating) based on observations made in the field and in a pilot study (Appendix I) (Table 1). "Predator avoidance" was comprised of three specific behaviors: self-burial, thanatosis, and shoot-climbing. Self-burial is effective in visually obscuring the billbug and may also obscure chemical cues predators might use for detection (Eastwood, 1996). Thanatosis can be distinguished from the billbug simply remaining motionless by the billbug tightly drawing in its legs to its body, often causing it to fall onto its side or back. Shoot-climbing behavior was classified as predator avoidance as it constitutes an alteration in space use that reduces feeding and mating opportunities, but that facilitates avoiding ground-dwelling predators. Such tradeoffs have been documented in other invertebrates (Brown and O'Connel, 2000; Lima and Dill, 1990; Sih, 1982). Taking a conservative approach, billbugs that were motionless on or walking across the soil surface were not classified as engaging in predator avoidance behavior, in part because those behaviors may serve additional purposes not related to predator avoidance. Though thanatosis is a well-documented behavior in billbugs as a response to disturbance, we did not observe this behavior in our trials, so graphs showing predator avoidance are a combination only of self-burial and shoot-climbing (Kindler and Spomer, 1986).

We documented the behavior of each of the four billbugs in each replicate every 10 minutes over the course of 70 minutes. The first observation occurred 10 minutes after billbug introduction to the arena for a total of 7 observations for each trial. At each data collection interval, each billbug of the four present was assigned a positive outcome (1) for the one behavior they were displaying, and a negative outcome (0) for all others. In the few cases where

in a billbug died during the 70 minute observation period, death was recorded as a positive outcome only once. The sum of all instances of each behavior (Table 1) recorded over the course of the trial was taken for each replicate (individual arena), and this frequency was used for data analysis.

2.2.3 Data Analysis

We used generalized linear models (GLMs) to compare frequency of behaviors between treatment conditions. The sum frequency of each behavior in each replicate served as a unique data point, with behaviors analyzed independently. Three Poisson GLMs were used to test for significant differences in frequency of 1) feeding, 2) predator avoidance behavior, and 3) mating in different predator and cue conditions (control, direct contact with predators, indirect contact with predators or non-predatory arthropods, and predator odor). To isolate the effect of predators generally, in the direct and indirect predator contact conditions we combined replicates from all predator treatments (both single predator species and predator species pairings) of that contact condition (indirect n=40, direct n=40) to be compared against a control (no predator) (n=8), predator scent (n=16), and non-predatory arthropod treatment (n=8). In the predator odor treatment, we combined replicates from all four predator odors (*P. melanarius, Harpalus* sp., *Philonthus* sp., and *Lycosid* sp.) as their effects on billbug behavior did not differ (n=16) (Table 2C). In the non-predatory arthropod treatment, we combined data from both arthropods (*Armadillidae* sp. and *Acrididae* sp.), as their effects on billbug behavior did not differ (z= -0.56, p=0.99) (n=4 reps/species).

To determine if direct contact with a single predator species versus a pairing of two predator species had different effects on 1) feeding or 2) predator avoidance we used two Poisson GLMs comparing our predator species richness conditions (0, 1, or 2). No predators

represented the control condition (n=8). Replicates from all four predators of study represent the single predator treatment (n=16), and all replicates from all six predator pairings were combined to constitute the predator pair treatment (n=24). The same two models were run for the indirect contact data.

To analyze the effect of species-specific interactions and billbug direct contact with each unique predator species and different predator pairings we used Poisson GLMs comparing the effect of each species or their pairings on 1) feeding and 2) predator avoidance behavior. We used the same models to compare the effects of indirect contact with predator species and species pairs on billbugs behaviors.

All models were fit using an offset of 28 to correct for the total number of possible observations for all four billbugs over the entire hour of testing, and using Tukey's honestly significant difference (HSD) posthoc test for multiple comparisons. Models were fit in RStudio (version 1.1.463) using the stats package (version 4.0.3) and pairwise contrasts were made using Tukey's HSD (MultComp package version 1.1.463).

2.3. Billbug Response to Predator VOCs Using Y-tube Choice Test

2.3.1 Treatments

This experiment was designed to determine if billbugs 1) can detect VOCs emitted from common predators and if so, 2) respond differently to VOCs from a single predator species versus predator pairings. In a series of Y-tube assays billbugs chose between filtered air in one arm of the Y-tube, and an arm containing headspace cues from predator(s) or a non-threatening arthropod (Fig. 2). For the single predator treatment, billbugs were presented with the cues from predators individually (*P. melanarius, Harpalus* sp., *Philonthus* sp., and *Lycosid* sp.) (20

reps/species; n=80). For the predator pair treatment, they were presented with all possible pairings of the four predators above (with the exception of the *Philonthus* and *Lycosidae* combination, which could not be completed due to lack of seasonal co-occurrence and general low abundance) (20 reps/pairing; n=100). A non-predatory arthropod treatment was employed to test for billbug reaction to VOCs from a heterospecific (*Armadillidae* sp.) that presents no consumptive threat (n=20). Our ability to replace the predators in the cue chambers was limited by some species' abundance, so one set of predators was used during half of the trials and a different set was used for the second half to increase the number of predator individuals to get a more representative sample of the predator population and to reduce impact of time the predator spent in the stressful environment of the cue chamber. In predator species richness of two treatments, the ordering of the predators in the cue chambers was reversed after 10 trials to account for impacts of upstream predator VOCs on the downstream predator.

2.3.2 Y-tube apparatus and experimental design

Y-tubes had an inner diameter of 1.5cm, arm length of 7.5cm, and stem length of 8.5cm (The Custom Glass Shop Glass and Plastic Labware, Vineland, NJ). Constant flow of charcoal-filtered air was maintained at 1 L/min into each arm of the apparatus using Buck air samplers (A.P. Buck, Orlando, FL). Tygon tubing sections with corks affixed to their ends were used to direct airflow into the arms of the Y-tube. Air was passed from the air sampler through two glass cylinders (inner diameter=1cm, length= 3.5cm), referred to as cue chambers hereafter, before entering either arm of the Y-tube (Fig. 2).

The influence of light and movement on billbug choice was minimized by placing each Y-tube setup in a white box, with only one side left open to make observations of billbug behavior. A full-spectrum OTT-LIGHT (Environmental Concepts, Tampa, FL) was placed

directly over the Y-tube apparatus. White paper was used to obstruct billbugs' view of

equipment in the box, such as the air sampler, base of the light and charcoal filter so that the

visual environment around the end of the apparatus containing cues was symmetrical and would

not impact decision (similar to Blackmer et al. 2004). The apparatus was rotated such that the

direction of the cue arm and the blank arm were reversed every 5 trials to control for any

directional bias. Tubing and corks were used only for a single predator treatment condition and

glassware was washed using Liquinox detergent, rinsed with acetone, and finally rinsed with

hexane between treatments.

Trials were conducted between 6/14/2019 and 8/9/2019, from 9:00 to 16:00 hours MST,

at room temperature. Billbugs were collected, stored and acclimated as described in section 2.1.

Each billbug was introduced into the base of the Y-tube, the end of which was then immediately

blocked so that the billbug could not escape. A 'choice' of either cue or blank arm was recorded

when a billbug entered either arm of the tube and passed 1cm down its length and remained

within that arm for at least 10 seconds. If no choice was made after 10 minutes the trial was

concluded and no choice was recorded.

2.3.3 Data Analysis

Chi-squared tests for independence were used to determine if the billbugs selected the

blank versus predator cue arm at different rates than would be expected (null hypothesis that

billbugs would select each arm with equal frequency) in the single predator species treatment and

in the predator pairing treatment. We also analyzed the no response rates, and the time before a

decision was made for each predator treatment (Blackmer et al., 2004) using binomial GLMs

with Tukey control for multiple comparisons (binomial and gaussian respectively). Chi-squared

tests and GLMs were run using the stats package (version 3.6.2), and the package multcomp was used for Tukey tests in RStudio (version 1.3.1093).

3. Results

3.1. Billbug behavior when exposed to direct and indirect predator cues in microcosms

No billbugs were successfully consumed by predators in this assay. There were, however 8 attacks on billbugs (3% of observations in the direct predator contact condition), always by one of the two carabids of study (*P. melanarius* or *Harpalus* sp.). Despite the lack of billbug consumption by predators, mean feeding frequency decreased in both direct predator contact ($z=$ -7.53, $p<0.001$) and indirect predator contact ($z=$ -4.11, $p<0.001$) treatments, as well as in the predator odor treatment ($z=$ -4.43, $p<0.001$) in comparison to the no predator control. Feeding frequency did not decrease significantly compared to the no predator control in the non-predatory arthropod treatment ($z=$ -2.42, $p=0.10$) (Fig. 3A). Billbugs in direct contact with predators showed the greatest decrease in mean feeding frequency (-77% compared to no predator control), a decrease significantly greater than all treatments ($[z]>3.97$, $p<0.001$) except for the predator odor treatment ($z=-2.61$, $p=0.077$). Billbugs in indirect contact with predators or exposed to predator odor also showed decreased feeding frequency compared to the no predator control (-50% and -61% respectively).

The mean frequency of billbug predator avoidance behaviors increased in the direct predator contact ($z=$ 5.51, $p<0.001$) and indirect predator contact ($z=$ 4.31, $p<0.001$) treatments, as well as in the predator odor ($z=$ 5.76, $p<0.001$) and non-predatory arthropod ($z=$ 4.78, $p<0.001$) treatments compared to the no predator control. Increases in these avoidance behaviors ranged from 75% when exposed to single predator species richness levels to as much as 116% when exposed to predator odor (Fig 3B). Though all treatments differed from the no predator

control, the only other treatments that differed from one another were the indirect contact and predator odor treatments (z= -3.02, p=0.019), while all other pairwise comparisons of treatments showed no significant difference ([z]>0.03, p>0.05).

Billbug mating frequency was extremely low in assays, only observed in 24 of 728 (3%) of observations (Fig 3C). The no predator control had the mean highest mean mating frequency of any treatment, significantly higher than in the direct and indirect predator contact treatment, and, than the predator odor treatment (z=-3.49, p=0.0029). Mating frequency in the non-threatening arthropod treatment did not differ from the no predator control (z=-0.015, p=1.00).

Billbugs responded to direct contact with one predator species (z=-6.01, p<0.001) and two predator species (z=-6.29, p<0.001) with significant decreases in feeding frequency compared to the no predator control, but did not show different feeding frequency in the one versus two predator species treatments (z=0.31, p=0.95) (Fig 4A). While billbugs also showed significant increases in predator avoidance behaviors in both the one predator (z=4.50, p<0.001) and two predator (z=6.55, p<0.001) treatments compared to the no predator control, they showed significantly higher rates of predator avoidance behaviors when directly exposed to two predator species than to one (z=3.30, p=0.0025) (Fig 4B). When billbugs were in indirect contact with predators, results differed. While the number of predator species present did not influence the magnitude of increase in predator avoidance behaviors (z=-0.26, p=0.96) (Fig 4D), feeding frequency of billbugs exposed to two predators was significantly lower than those exposed only to one predator species (z=-3.30, p=0.0028) (Fig 4C). The indirect contact condition also differed in that feeding frequency was not significantly different from the no predator control when only one predator species was present (z=-2.11, p=0.086) (Fig 4C).

Though billbugs sometimes responded more strongly to predator pairings than to single predator species, no single predator or predator pairing consistently induced the strongest behavioral response (Fig 4). When in direct contact with billbugs, no predator differed from any other in its influence on billbug feeding or predator avoidance behaviors, nor did any predator pairings differ in their relative effects (Table 2A). Some difference in effect of predators and predator pairings were observed in the indirect contact treatment, with billbugs indirectly exposed to *Harpalus* sp. showing greater feeding frequency than those indirectly exposed to *Lycosid* sp., and billbugs indirectly exposed to *P. melanarius* showing greater frequency of predator avoidance behaviors (Table 2B). Indirect contact with all predator pairings had the same influence upon feeding frequency. The pairing of *Lycosid* sp. and *P. melanarius* induced a larger increase in predator avoidance behaviors than did the pairing of *P. melanarius* and *Harpalus* sp., but all other pairings had a similar effect on predator avoidance behavior frequency (Table 2B).

3.2. Billbug Response to Predator VOCs Using Y-tube Choice Test

Billbugs selected the arm containing no predator VOCs in 68.3% of trials compared to the arm containing predator VOCs in 31.7% of trials (null of equal selection frequency; $X^2 = 21.95$, df= 1, p<0.001). Billbug choice for no predator VOCs was higher for both single predator species ($X^2 = 11.71$, df= 1, p<0.001), and predator species pairs ($X^2 = 10.27$, df= 1, p<0.001). (Fig. 5). Billbugs did not differentiate between VOCs from non-predatory arthropods and filtered air ($X^2 = 0.60$, df= 1, p=0.44). Neither the rate at which billbugs failed to respond (44% overall) (t<1.93, p<0.001) nor the time before a choice of cue arm was made (t<2.00, p<0.001) differed between our predator treatments.

4. Discussion

The resident predator community in turfgrass has recently been shown to contribute very little to the suppression of billbugs through direct consumption (Dupuy and Ramirez, 2019). Despite this, some of the most abundant predators in that community, namely *Pterostichus melanarius*, *Harpalus* sp., *Philonthus* sp., and *Lycosid* sp., cause risk-induced trait responses in billbugs. Specifically, direct exposure to predators led billbugs to decrease their feeding and mating frequency (by 77% and 94% respectively), and increased frequency of predator avoidance behaviors (160%) (Fig 4). Such effects on behavior are extremely well documented (Buchanan et al., 2017; Lima and Dill, 1990; Nelson et al., 2004; Rendon et al., 2016; Rypstra and Buddle, 2013; Tan et al., 2013; Werner and Peacor, 2003; Williams and Wise, 2003), and ours is not the first study to find a lack of correlation between actual risk of predation and magnitude of prey risk-induced behavioral responses (Kunert and Weisser, 2003; Pessarrodona et al., 2019; Relyea, 2001; Trussell et al., 2011). In fact, similar to billbugs, Colorado potato beetle (*Leptinotarsa decemlineata*) adults are invulnerable to predation by spined soldier bugs (*Podisus maculiventris*), but also alter behavior (i.e. reduce feeding) in the presence of those predators (Hermann and Thaler, 2018).

We found evidence that billbugs use chemical cues to detect predator presence and modulate those observed behavioral responses. Not only did billbugs respond similarly when exposed directly to predators and when presented with predator odor alone (Fig 3), but they also significantly avoided predator VOCs in Y-tube experiments ($p<0.05$) (Fig 5). Induction of behavioral change and NCEs via predator-associated chemicals has been shown in other insect systems (Aflitto and Thaler, 2020; Hermann and Thaler, 2014). Though ours is the first study to suggest predator VOCs induce NCEs in billbugs, previous work in hunting billbugs has

demonstrated their ability to employ chemical cues, specifically those of conspecifics, to navigate their environment (Barrett et al., 2018; Duffy et al., 2018). While we found evidence that billbugs sense predator-associated chemicals, recognition and response to these cues is not predator species specific. Though overall billbugs did respond more strongly to predator species pairings than to single predator species, they did not respond differently to different predator species, or different pairings (Table 2; Fig 4). Billbugs also did not respond more strongly to the only predators that were documented attacking adults during assays (*P. melanarius* and *Harpalus* sp.), or predator pairings containing either or both of these carabids. Though these two predator species are more likely to attack, none of the predators pose a significantly higher direct consumptive threat than any other tested herein, so a lack of predator species differentiation by billbugs in this study would perhaps be expected. Considering this, it was surprising to see these behavioral responses at all, in the absence of a substantial consumptive threat. It may be that responses observed here are an adaptation to avoid other, more effective predators. Billbugs do have other predators not examined here —both other arthropods (e.g. ants) (Dupuy and Ramirez, 2016) and vertebrates (e.g. birds and amphibians) (Young, 2002)— that these defenses may be better suited for. Alternatively, adults could be responding to predators that pose a risk to eggs, a stage that appears to be much more susceptible to predation, particularly by ants, a predator not studied here (Dupuy and Ramirez, 2019). Behavior of adult females often serves primarily to protect offspring more susceptible to predation, so further studies of oviposition preference in the presence of predators are warranted (Höller et al., 1994; Jaenike, 1978; Munga et al., 2006; Stav et al., 1999; Vonesh and Blaustein, 2010).

Alternatively, billbugs' lack of differentiation between predators, and response to predators that pose little threat could also occur as a result of generalization of predator cues.

'Generalization of predator recognition' refers to prey's use of cues from known predators to identify novel predators (Ferrari et al., 2007). Such generalization of predator recognition can lead to misidentification of non-threats as a threat if there are cues common among threatening and non-threatening species. Though many organisms are able to identify unique predator species through use of species-specific cues, and modulate their response based on the threat posed by those predators (Chivers et al., 1996; Kusch et al., 2004), there is also much evidence of generalization of predator cues in vertebrates (Ferrari et al., 2008, 2007; Webb et al., 2010), as well as in invertebrates (Rochette et al., 1997). Insects (specifically mosquito larvae *Culex pipiens*) have also been shown to respond to general disturbance cues with antipredator behavior when they lack the ability to identify specific predator species (Sih, 1986). Developing associations between cues and threats, and then generalizing across contexts necessitates a certain capacity for flexible learning, a capacity directly demonstrated in insects like the larval damselfly *Enallagma boreale* (Wisenden et al. 1997). Thus, insects have shown the ability to generalize disturbance (e.g. movement) and chemical cues, potentially explaining the similarity in billbugs' response to different predators if those predators share key cues identified by billbugs. Other studies have shown that some generalized predator cues derive from digested prey metabolites (Ferrari et al., 2007; Mortensen and Richardson, 2008; Smee and Weissburg, 2006), so our study in which predators were starved may even underestimate the magnitude of NCEs if such cues are used by billbugs (Weissburg et al., 2014; Weissburg and Beauvais, 2015). Further study could examine the chemical constituents of predator headspace volatiles and cuticular compounds to determine if there are compounds common amongst predators that may induce generalization of those cues, as well as the role of predator diet in induction of NCEs in billbugs. While responses to visual predator cues appear to be innate, responses to chemical cues

have been shown to be plastic and learned in many systems (Kelley and Magurran, 2003; Laurila, 2000), so understanding the effects of long term exposure, at densities experienced by billbugs in the field, will also be key.

The presence of a non-predatory arthropod elicited increases in predator avoidance and reductions in mating compared to the control (Fig 3). In fact, billbugs responded to a non-predatory arthropod being in the upper chamber of the experimental arena at times similarly to how they responded to a predator being in the upper chamber. Though some insects do "eavesdrop" on the alarm pheromones of heterospecifics to avoid danger (Wang et al., 2016), this billbug response to non-predatory arthropods was not likely a result of such chemicals, as billbugs did not avoid non-predatory arthropod VOCs in Y-tube experiments ($X^2 = 0.60$, df= 1, p=0.44). Instead, one possibility is that the presence of visual or vibrational cues from the non-predatory arthropod induced their response. Vibrational cues from an approaching predator, even in the complete absence of visual cues from that predator, can induce defensive responses in insects (Gish, 2021). Though some insects can differentiate the vibrational cues from predators versus non-predators (Gish, 2021; Sih, 1986), other insects lack that capacity to differentiate, and respond more generally to disturbance cues (Sih, 1986). The similarity in billbugs' response to the non-predatory arthropod and predator in the upper chamber of the arena suggests that they perceived non-predatory species as a threat similar to predator species. Thus, they do not appear to differentiate disturbance cues from predators and non-predators, and instead identify disturbance cues as a threat. Again, it appears that billbugs are generalizing cues that may indicate the presence of some real threat (effective predator) to situations in which similar disturbance cues are present but with no effective predator. Further isolation of different cues to determine the precise mechanism will be a logical next step, potentially providing key insight for

the development of pest management technologies that employ cues such as vibrations and acoustics (Aflitto and Hofstetter, 2014; Polajnar et al., 2015; Takanashi et al., 2019).

Billbugs generally responded more strongly to predator pairings than to single predator species in microcosm assays, with lower feeding and higher predator avoidance when predators were paired than in single predator treatments (Fig 4B-C). It is not likely that this results from sensing both species uniquely in their chemical cues as billbugs did not avoid VOCs of predator pairs significantly more than those from single species in Y-tube assays (Fig 5). Instead, it may be that the combination of different cues resulting from different predator species, with different behavior and ecology, is what induces the strongest behavioral response. Predator traits (i.e. hunting mode, size, etc.) influence prey risk-induced trait responses (Wirsing et al., 2021) so exposure to multiple predators, threatening to billbugs in different ways, may lead to stronger billbug responses in the presence of multiple predators. Again, though none of these predators pose a significant threat, the presence of multiple species might introduce a wider variety of cues through different behaviors and chemicals (Hazlett and McLay, 2005), and lead to stronger billbug responses. This integration of multiple cues inducing a stronger response is further supported by the fact that billbugs responded most strongly to direct predator contact, when they were exposed to all potential cues associated with predator presence (chemical, visual, vibrational, auditory, etc.) (Fig 3). This finding corroborates others' that suggest the importance of integration of multiple cues such as chemical and visual cues (Chivers et al., 2001; Stauffer and Semlitsch, 1993). It is worth noting that predators in pairings often attacked one another during microcosm trials, so the increased activity resulting from these conflicts may also explain the greater behavioral responses observed in some predator pair treatments. However, this deserves further study as both increased and decreased prey response to multiple predator

presence have been documented (Sih et al., 1998). Billbugs' predators do not kill them outright, as effective predators might. This may result in longer periods of risk and stress-induced behavioral change, and thus stronger NCEs (Trussell et al., 2011; Wirsing et al., 2021). Pulsed risk environments actually induce stronger NCEs than do high risk environments (Trussell et al., 2011), so the lack of serious consumptive threat from the most abundant predators that billbugs encounter most frequently may actually yield stronger NCEs than if these predators were extremely lethal. However, this assumes that billbugs do infrequently encounter more effective predators or more lethal threats. Rates of mortality in the field (6%) (Dupuy and Ramirez, 2019) were marginally higher than those we observed, 0% here, and 0.2% in pilot studies (Appendix I), so other predators may be at work, or predators of study may be marginally more lethal in a field setting.

Interestingly, billbugs did not display all of their known predator defense responses during the course of this study. For instance, though billbugs and many other weevil species display thanatosis (playing dead) in response to disturbance, we did not observe this behavior in response to contact with predators in our assays. Though billbugs routinely displayed thanatosis in response to being picked up with fingers or forceps (often remained motionless, with their legs tucked to their body for many minutes) it is difficult to determine what induced this response then, and why proximity to and contact with predators did not consistently induce the same response. This is the first study that we are aware of that documents billbugs partially burying themselves head-first in the soil for purposes other than overwintering. This behavior has been documented as a predator-defense and camouflage response in insects (i.e. chironomid larvae) (Hölker and Stief, 2005) and in other invertebrates (Aizaki Yoichi Yusa, 2009; Domínguez and Jiménez, 2005). Similarly, we are unaware of prior documentation of the shoot-climbing

behavior we observed here in billbugs, though it has been suggested to serve predator avoidance purposes in other arthropods (Brown and O'Connel, 2000; Schonewolf et al., 2006). Overall, our findings suggest that key questions concerning drivers of billbug behavior, and the purposes they serve remain unanswered, and that perhaps not all of billbugs' apparent defensive responses are intended for predators tested in this study.

5. Conclusions

Though predators pose little consumptive threat to billbugs, their presence and the presence of their cues could induce NCEs and benefit efforts to control billbug populations. Many studies have shown NCEs to be as or more impactful than consumption by predators (Křivan and Schmitz, 2004; Peacor and Werner, 2001; Thaler and Griffin, 2008; Trussell et al., 2006), and even insects that suffer little direct consumption show behavioral shifts as a result of predator presence (Hermann and Thaler, 2018; Schmitz, 1998). These individual changes in behavior may scale up and influence distribution of individuals and community structure overall (Matassa and Trussell, 2011), so fostering a healthy, diverse predator community could benefit IPM programs for billbugs. Billbugs response to non-predatory arthropods also add to the body of literature suggesting that a healthy and diverse community of non-predators may also be beneficial as well. NCEs appear to be stronger in more speciose, reticulate food webs (Schmitz, 1998), and non-enemies may increase suppression effects of predators (Ingerslew and Finke, 2018). Building tools beyond conservation biocontrol will necessitate gaining a more nuanced understanding of cues that induced anti-predator responses in billbugs in this study. Identification of cues that billbugs generalized to indicate the presence of a threat across predators and cue treatments could provide avenues for novel chemical and mechanical control techniques. Our results also have

implications for biocontrol research in other settings, particularly as pilot studies suggest that

billbugs respond similarly when in their natural species complexes (Appendix I). Given that

billbugs do not modulate their responses based on consumptive threat posed, a phenomenon

demonstrated in other systems as well (Kunert and Weisser, 2003; Pessarrodona et al., 2019;

Relyea, 2001; Sih, 1986), our work suggests a more comprehensive view of predator-prey

interactions, looking beyond predators' direct consumptive efficacy, is necessary when counting

on predator communities for biological control of pests. Though our study and others provide

compelling evidence for the existence and strength of NCEs, additional studies should also

examine multitrophic effects of NCEs to determine if they translate to decreased damage to

plants.

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Table 1. Observed behaviors in billbugs and their descriptions.

Global Behavior	Specific Behavior	Behavior Description
Mating	Mating	Active entry of aedeagus into female terminalia.
Feeding	Feeding	Active feeding upon plant tissue. "Active" feeding was defined as instances when rostrum was observed to be inside plant tissue or mandibles observed to be actively chewing plant material.
Predator Avoidance	Burial	Billbug observed to have partially buried itself with at least the entire head capsule, and up to the entire body, below the soil surface. Almost always the billbug was oriented vertically, with head facing downward, when this behavior was recorded.
	Thanatosis	Billbug "playing dead" by drawing in legs under its body.
	Shoot-climbing	Billbug climbing or residing on any plant structure above the crown of the plant (i.e. on a stem or blade).

Table 2. GLM results of microcosm assays comparing the frequency of billbug feeding and predator avoidance when exposed directly (A) or indirectly (B) to single predators or predator pairs or to single predator species' odors (C). Predator species abbreviations (Pt= *P. melanarius,* H = *Harpalus* sp., Ph= *Philonthus* sp., and L = *Lycosid* sp.). Significance p values (p<0.05) are noted in bold.

(A) Direct Contact				
	Feeding		**Predator Avoidance**	
Single Predators	z	p	z	p
L-H	1.22	0.60	-0.093	1.00
Pt-H	0.00	1.00	0.54	0.95
Ph-H	1.64	0.35	0.45	0.97
Pt-L	-2.23	0.60	0.64	0.92
Ph-L	0.50	0.96	0.55	0.95
Ph-Pt	1.65	0.35	-0.089	1.00
Predator Pairs				
HPt-HL	-0.98	0.862	-0.24	0.99
LPt-HL	0.77	0.94	0.31	0.99
LPh-HL	-0.30	0.99	-0.82	0.93
PrPh-HL	0.28	0.99	-1.16	0.78
LPt-HPt	1.65	0.46	0.55	0.98
LPh-HPt	0.70	0.96	0.58	0.89
PrPh-HPt	1.23	0.73	0.91	0.80
LPh-LPt	-1.05	0.83	-1.13	0.79
PtPh-LPt	-0.5	0.99	-1.47	0.58
PtPh-LPh	0.58	0.98	-0.034	0.99

(B) Indirect Contact				
	Feeding		Predator Avoidance	
Single Predators	z	p	z	p
L-H	-2.68	**0.036***	-1.32	0.55
Pt-H	-1.5	0.44	1.26	0.58
Ph-H	-1.83	0.25	-1.62	0.36
Pt-L	1.33	0.53	2.55	0.052
Ph-L	0.99	0.75	-0.31	0.99
Ph-Pt	-0.35	0.98	-2.84	**0.023***
Predator Pairs				
HPt-HL	0.00	1.00	-0.99	0.86
LPt-HL	0.99	0.86	1.83	0.35
LPh-HL	1.57	0.51	-0.096	1
PrPh-HL	1.19	0.75	1.08	0.81
LPt-HPt	0.99	0.88	2.79	**0.042***
LPh-HPt	1.57	0.51	0.90	0.89
PrPh-HPt	1.19	0.75	2.06	0.23
LPh-LPt	0.62	0.97	-1.92	0.31
PtPh-LPt	0.22	0.99	-0.75	0.94
PtPh-LPh	-0.41	0.99	1.18	0.76

(C) Predator Odor				
	Feeding		Predator Avoidance	
Single Predators	z	p	z	p
L-H	-0.21	0.99	0.46	0.97
Pt-H	-1.90	0.22	-1.74	0.30
Ph-H	0.20	0.99	-1.83	0.26
Pt-L	-1.73	0.30	-2.19	0.18
Ph-L	0.41	0.99	-2.27	0.10
Ph-Pt	0.57	0.16	-0.09	1.00
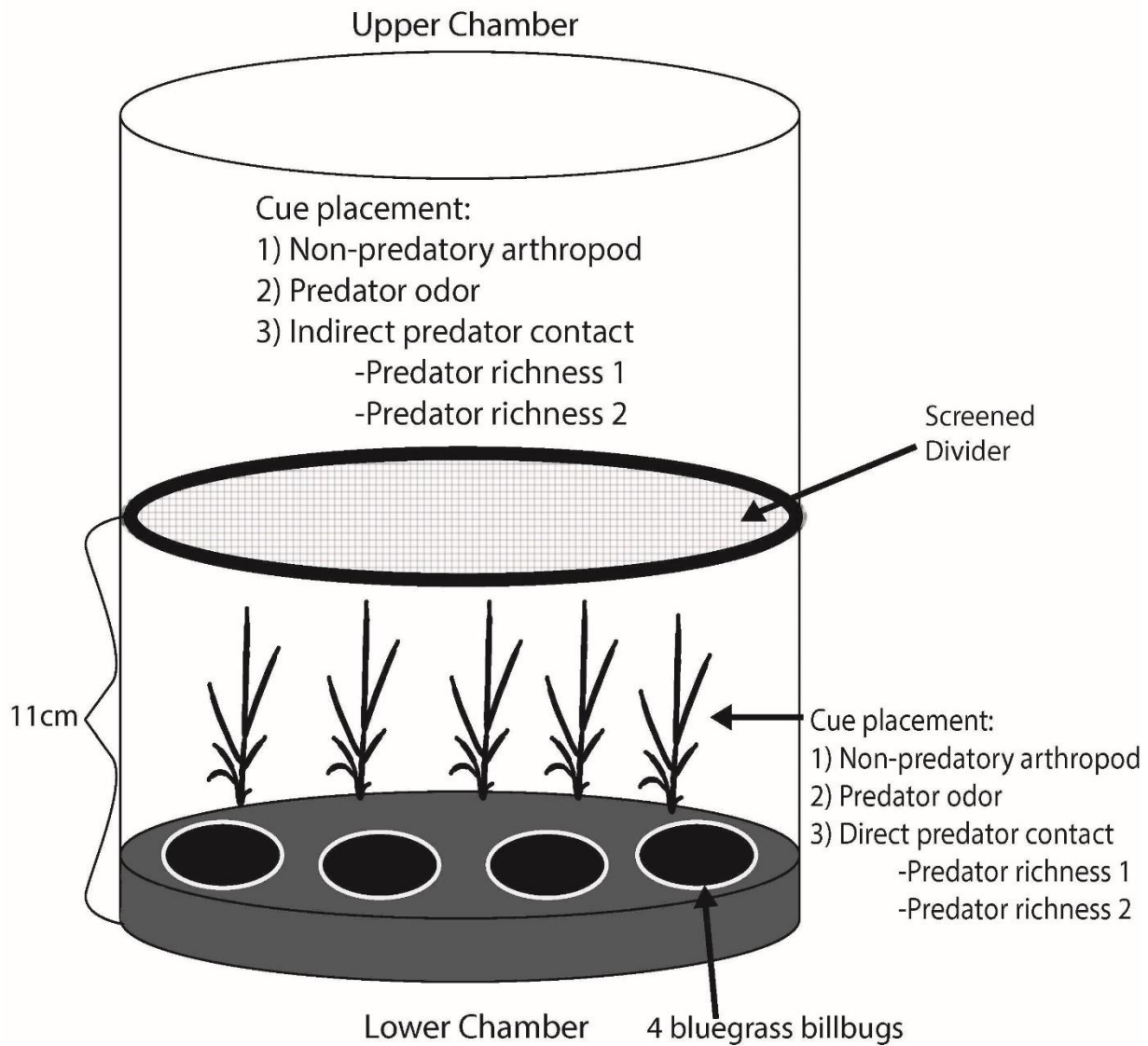
Figure 1: Modified Bioquip mosquito breeders (21 x 12 cm) consisting of an upper and lower chamber (clear, quart-sized styrene containers) separated by a screen were used for microcosms. The lower chamber contained field soil substrate 2cm deep planted with five Kentucky bluegrass stems and four billbugs in all replicates. In direct contact replicates predators were also placed in the lower chamber. In indirect contact replicates predators were placed in the upper chamber. In positive control replicates (non-predatory arthropod and predator odor) cues were placed in the upper chamber.

Charcoal Filter

Air Pump

Airflow

Airflow

Pred

Pred

Muslin covers over each cork
(not shown)
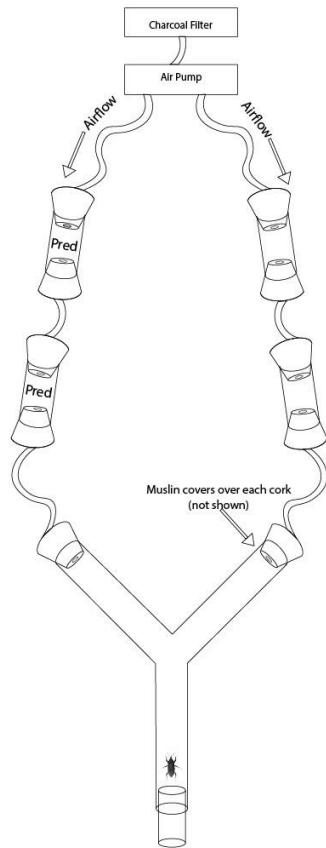
Figure 2: Y-tube apparatus used in assays had an inner diameter of 1.5cm, arm length of 7.5cm, and stem length of 8.5cm. Charcoal filtered air was pumped into the apparatus (top), through Tygon tubes, into the cue chambers (either containing a single predator, or empty), and finally into the arms of Y-tube. For each trial a single billbug was placed in the base of the Y-tube and allowed 10 minutes to select an arm of the apparatus.

Figure 3: Frequency of observed feeding behavior (A), predator avoidance behavior (B), and mating (C) by billbugs per replicate (two- chamber arena) while billbugs were in control, non-predatory arthropod, predator odor, indirect and direct contact conditions. Conditions assigned different letters indicate significantly different behavioral frequency between conditions ($p < 0.01$). N=112.

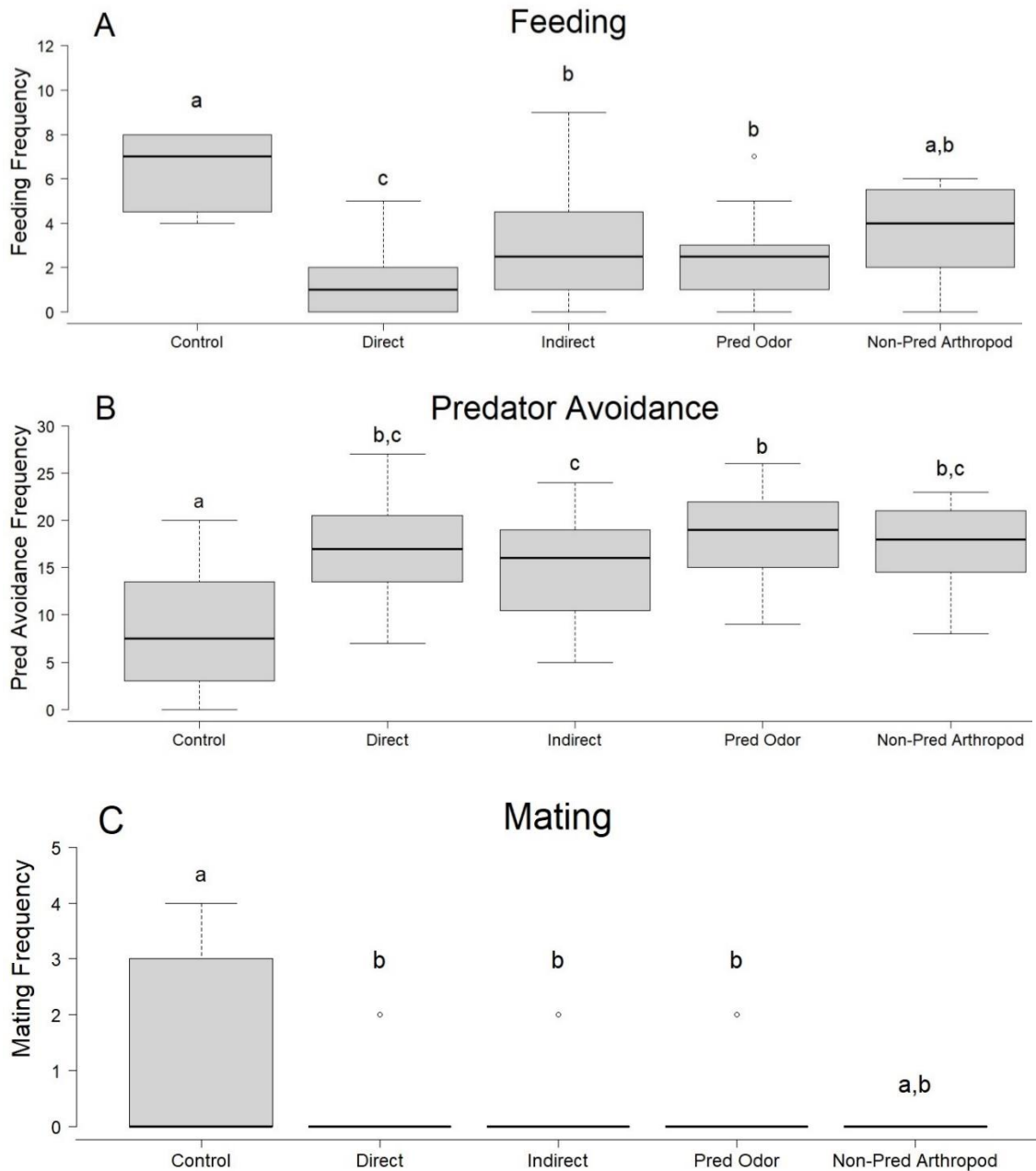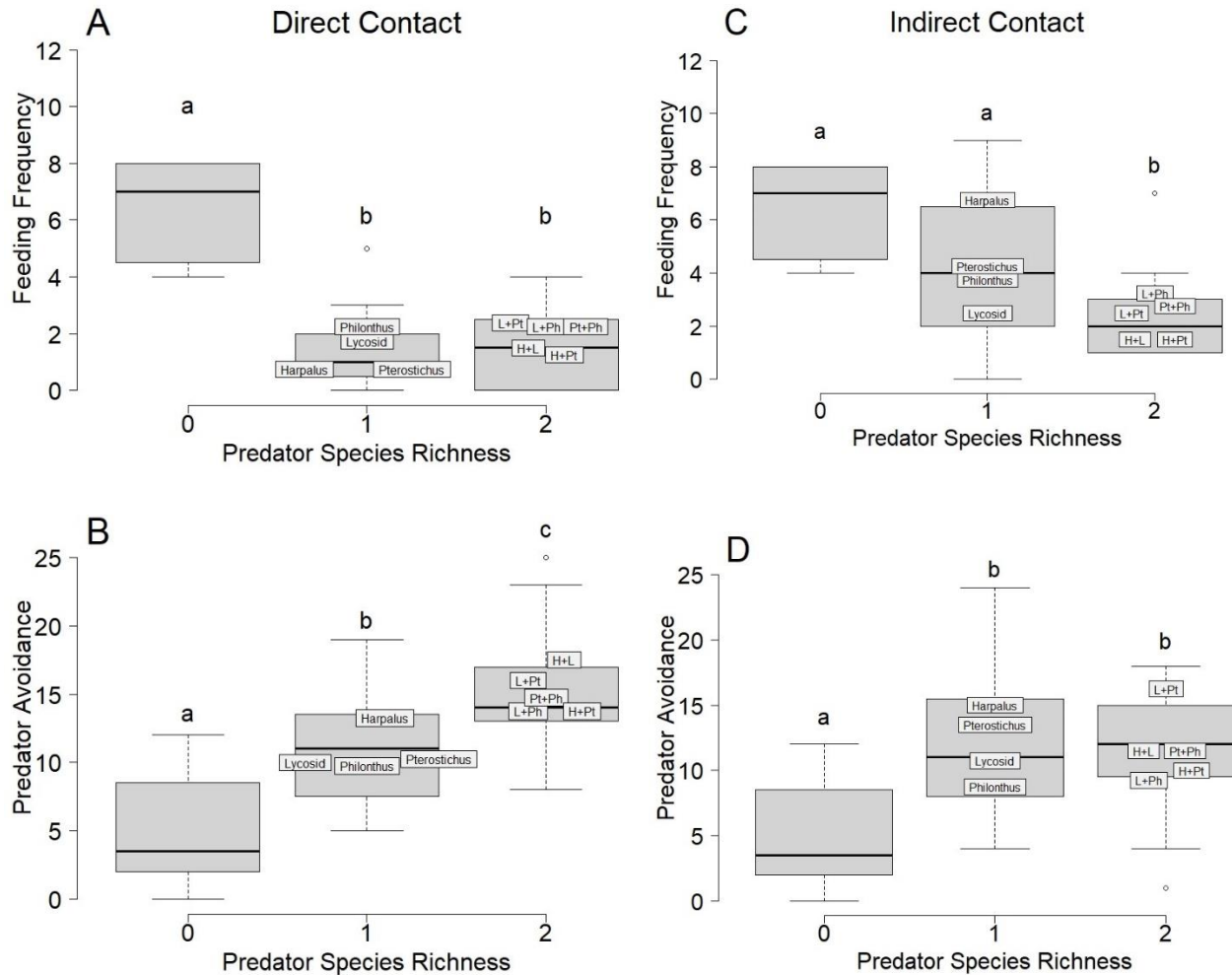**Figure 4:** Count of billbug feeding events observed in each replicate (2 chamber arena) where billbugs were in direct contact (left) (n=48) and indirect contact (right) (n=48) with 0, 1, or 2 species of predators (n=8, 16, 24 respectively). Upper plots show feeding behaviors, while lower show predator avoidance behaviors. Different lowercase letters indicate significantly different behavioral frequency between conditions (p< 0.05). Mean frequency of each behavior, in each contact condition represented by boxes with predator species names or with a combination of abbreviations in predator species richness 2 treatments (Pt= *P. melanarius,* H = *Harpalus* sp., Ph= *Philonthus* sp., and L = *Lycosid* sp.)

Figure 5 Proportion billbugs selecting blank arm (black bars) versus selecting cue arm with non-predatory arthropod (NT) (n=15), one predator (P1) (n=51) or two predator (P2) (n=62) species VOCs (grey bar). Within treatment difference from Chi-squared expected 50:50 proportion indicated by ** X^2 p <0.01, *** X^2 p <0.001.

CHAPTER III

BLUEGRASS BILLBUG (SPHENOPHORUS PARVULUS) HOST PREFERENCES FOR
KENTUCKY BLUEGRASS WITH VARIED IRRIGATION AND DROUGHT RESISTANCE
LEVELS

Abstract

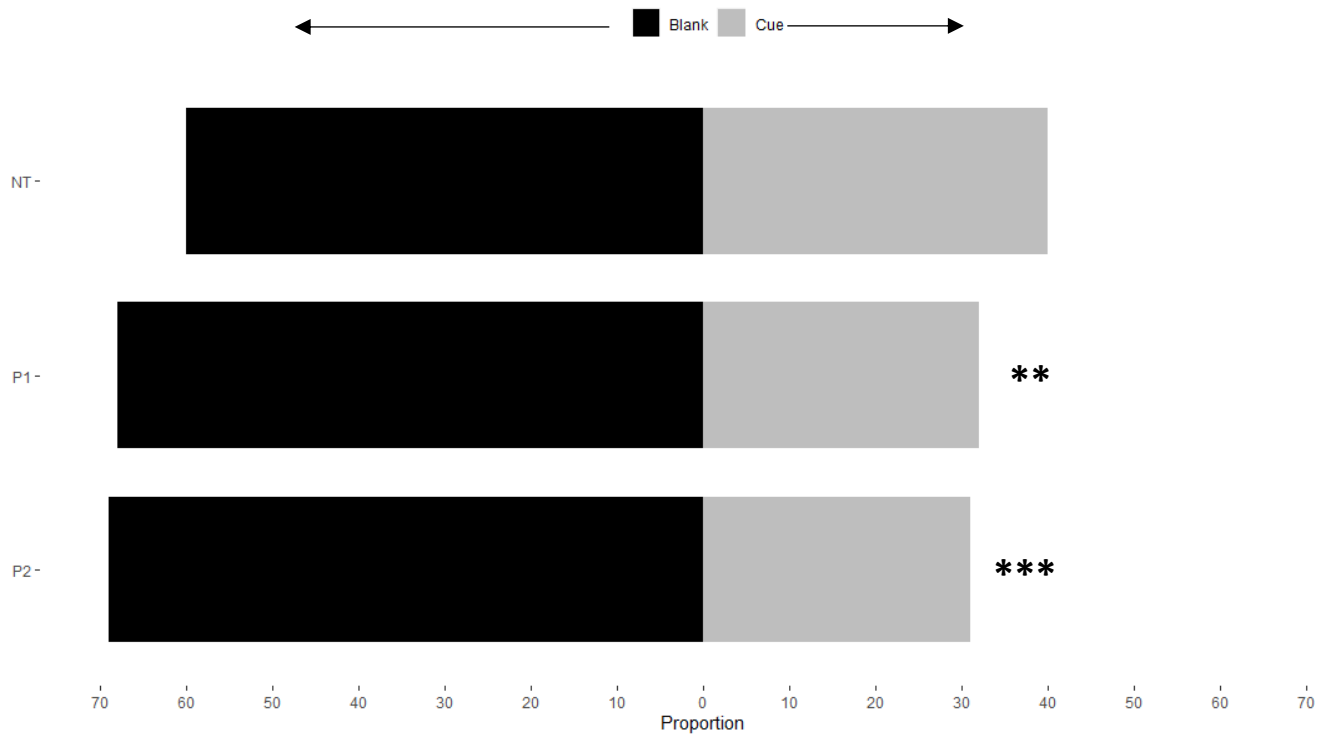Drought resistant Kentucky bluegrass (KBG) cultivars have been developed to reduce the impact of water limitation, a common phenomenon in the Intermountain West. However, the influence of drought resistance on host preference and resistance to a key turfgrass pest, the billbug (*Sphenophorus* sp.), warrants investigation. Billbug damage is most apparent in turfgrass suffering from water-stress; but it is unclear whether billbugs select water-stressed areas, or if incidental co-occurrence of billbug and water stress leads to overall higher plant damage. To determine the relationship between billbug activity and soil moisture we first recorded adult billbug abundance at pitfall traps located within four golf courses in Utah. We found significantly greater billbug abundance in areas with lower soil moisture. Next, we evaluated the relationship between drought resistance and billbug resistance in 349 KBG cultivars using National Turfgrass Evaluation Program data. We found that increased cultivar drought resistance was correlated with lower billbug damage. We followed these results with a series of adult billbug choice assays to determine the extent to which these patterns were due to billbug host preference. We evaluated adult bluegrass billbug (*S. parvulus* Gyllenhal) preference for four KBG cultivars (Award, Baron, Jump Start and Midnight) with different drought resistance traits, as well as billbug preference when those cultivars were exposed to optimal or water-limited irrigation. While billbugs did not show a preference for drought susceptible cultivars or water-limited plants, they did show cultivar preferences. However, these preferences were not for

cultivars that were most drought susceptible or cultivars that suffered the most severe billbug damage, suggesting adult preference is not driven by drought resistance, and that adult preference does not correlate with level of billbug damage sustained by different turfgrasses. Though billbugs are found in greater abundance in drier areas, and more severely impact less drought tolerant KBG cultivars, this does not appear to be a function of host selection by adult billbugs based on the water-limitation or drought resistance of hosts.

Key words: host selection, Y-tube, turfgrass, drought stress

Introduction

Both the frequency and severity of drought events have begun to intensify throughout the Western United States and forecasts indicate that soil moisture deficits will likely continue to worsen in coming years (Cook et al. 2018, Williams et al. 2020). These spells of hot and dry weather, coupled with irrigation bans imposed to conserve limited water resources may severely impact turfgrasses, causing levels of damage that are aesthetically problematic, and environmentally and economically costly. Cool-season turfgrasses are particularly prone to high rates of dormancy under such heat and drought stress conditions. The most widely used cool-season turfgrass in the United States is Kentucky bluegrass (KBG) (*Poa pratensis* L.), common in lawns, golf courses, athletic fields, and parks (Brooks 2004, Lyman et al. 2007, Bushman et al. 2012). Though KBG is often thought of as a poor performer under water-limited conditions, it is highly adaptable, showing a wide range of drought resistance traits (Johnson-Cicalese et al. 1990, Funk 2000, Richardson et al. 2009, Lewis et al. 2012). Hundreds of KBG cultivars have been developed with unique adaptations and traits that increase drought resistance, and are commercially available for use by managers looking to improve performance and appearance of their turf (Johnston and Johnson 2000).

In the Intermountain West, billbugs (*Sphenophorus* sp.) are one of the predominate insect pests of turfgrass, with the bluegrass billbug (*S. parvulus* Gyllenhal) being the most common managers contend with (Dupuy et al. 2017). Billbug larvae feed on the roots and crowns of turfgrasses, hollowing out stems and severing the roots from the aboveground tissue, causing the spreading of brown turfgrass patches in midsummer. Billbug infestation is one of the most often misdiagnosed turfgrass afflictions, commonly being mistaken for drought stress (Niemczyk 1983). On one hand, misdiagnosis of billbug stress may result from temporal overlap, with

billbug and drought stress manifesting simultaneously in the late summer (Niemczyk 1983), and the challenges in diagnosing cryptic, soil-dwelling billbug larvae as the root problem. On the other hand, the co-occurrence of billbug and water stress may result from plant-arthropod interactions. Managers and researchers have long noted that billbug damage appears to be worst in areas suffering from drought stress (Shetlar and Andon 2012); but whether it is drought stress that fosters billbug outbreaks, or billbug outbreaks that stress turfgrass and make it more prone to drought stress remains unknown.

Plant water-stress can lead to outbreaks of herbivorous insects through a variety of mechanisms: accumulation of soluble carbohydrates and essential amino acids may benefit insects nutritionally (Brodbeck and Strong 1987, Shen et al. 1989, DaCosta and Huang 2006), increased leaf surface or canopy temperatures favor population growth (Throssell et al. 1987), and drought stress causes decreased levels of some defense compounds (Gutbrodt et al. 2011). Insect herbivores may be able to identify drought-stressed hosts as superior, and do show preferences based on drought-stress (Showler and Moran 2003, Gutbrodt et al. 2011). Drought-stressed and well-watered plants often have distinctive volatile organic compound (VOC) profiles (Branco et al. 2010, Šimpraga et al. 2011) that phytophagous insects use to locate and select host plants (Bruce et al. 2005, Magalhães et al. 2018). Considering that water stress can alter host plants and that these changes are favorable to insect herbivores, it is important to understand these interactions within the framework of pest outbreaks and integrated pest management.

To alleviate abiotic stresses, turf breeders have developed drought tolerant and drought resistant cultivars (Bushman et al. 2012). These same plants may assist in management of biotic stressors, with reduced pest density documented on drought-resistant cultivars (Ruckert et al.

2021). Drought resistant turfgrasses tend to have physiological and morphological traits such as thicker epicuticular wax, tougher leaf tissue, and reduced root volume in the upper 10 cm of soil, in favor of deep root development (Marcum et al. 1995, Bonos and Murphy 1999, Huang and Gao 2000, Jiang and Huang 2001, Richardson et al. 2008, Kopp and Jiang 2015). Thicker and tougher plant tissue, and increased defense compounds resulting from drought stress have been shown to suppress insect feeding (Raupp 1985, Foggo et al. 1994, Rejeb et al. 2014, Kuglerová et al. 2019), and decreased root volume at shallow depths may be detrimental to billbugs' root-feeding larvae. There is also evidence of molecular cross-resistance for abiotic and biotic stressors, suggesting the possibility of cross resistance between drought stress and billbug stress (Ramegowda et al. 2020). Crosstalk in the signaling pathways induced by abiotic and biotic stress —potentially involving phytohormones, transcription factors, kinase cascades, and reactive oxygen species (ROS)— may lead to synergistic responses and cross-resistance to these different stressor types. Together, physiological, morphological, and molecular characteristics of drought resistant turfgrass plants appear likely to reduce their favorability as hosts for billbugs. However, different herbivore species show very different responses to drought-stressed and drought resistant plants, so it is important to understand how billbugs specifically respond in order to inform cultivar selection and cultural management tactics (Preszler and Price 1995, English-Loeb et al. 1997, Gutbrodt et al. 2011, Chidawanyika et al. 2014).

Billbugs have shown preferences for different turfgrasses species, and levels of billbug damage differ among turfgrass species (Johnson-Cicalese et al. 1989, Johnson-Cicalese and Funk 1990, Barrett et al. 2018, Duffy et al. 2018). Fine scale, genotype and cultivar-level difference in billbug damage have also been shown in zoysiagrass (Fry and Cloyd 2011) and KBG (Ahmad and Funk 1982). However, from these studies, it is not clear if these differences are a result of

greater billbug abundance due to host selection, or different susceptibility of plants to billbug damage. The link between specific plant traits and billbug host preference and damage is also lacking in turfgrass evaluations. Here, we present the first study we are aware of examining the influence of drought-stress and drought tolerance on billbug host preference and level of billbug damage.

We conducted a series of surveys and behavioral assays to determine if billbugs co-occur with water-stress in turfgrass, and if this could be due to adult billbug host selection for drought stressed KBG and/or drought susceptible KBG cultivars. First, we conducted a survey of soil moisture and adult abundance at four golf courses in Utah to determine if billbug abundance is higher in drier areas, that may be suffering from drought stress. We also conducted an analysis of National Turf Evaluation Program (NTEP) data on 349 KBG cultivars to determine if cultivars with greater drought resistance displayed lower levels of billbug damage. We then used choice assays to determine if patterns observed in the field may result from adult billbugs selecting for water-limited plants, or cultivars with lower drought resistance.

Materials and Methods

Survey 1: Evaluation of adult billbug abundance relative to field conditions

Soil temperature and moisture, and billbug abundance were monitored at 18 linear pitfall traps (as described in Dupuy and Ramirez 2016) in the roughs of 4 different golf courses during the summers of 2019 and 2020. Six traps were located at Logan Country Club in Logan, Utah (41.7661, -111.8107) and four traps at each of three municipal golf courses located near Salt Lake City, Utah: Rose Park (40.8006, -111.9310), Nibley (40.7116, -111.8750), and Forest Dale

(40.7185, -111.8632). All of these courses have roughs of well-established, sprinkler-irrigated *Poa pratensis* L. and soil of predominately silty loam. The Logan Country Club, the only private course in our survey, has successfully managed its billbug population over the past few years with localized applications of imidacloprid. The municipal courses (Rose Park, Nibley and Forest Dale) have a limited budget for pest management and did not treat for insect pests over the course of this survey.

Weekly collection from all traps occurred during June and July, and then bi-weekly through the end of August of 2019. In 2020, weekly collection only occurred at the three municipal courses in Salt Lake City from the beginning of May through the end of June, due to the COVID19 pandemic. In both years sampling captured the period of greatest adult activity (which was delayed in 2019 due to cold, wet weather) and was ongoing during peak activity, which occurs around mid-June in Utah (Dupuy et al. 2017). The time and ambient temperature were recorded at each collection. Soil temperature and volumetric water content (VWC) in the upper 4 inches of soil were also recorded as the average of 3 measurements taken around each trap using a FieldScout TDR 100 Soil Moisture Meter (Spectrum Technologies, Aurora, IL). We used these data to determine correlation between adult billbug abundance and soil moisture and soil and ambient temperature.

Survey 2: Evaluation of billbug damage ratings relative to cultivars with varied drought resistance

To determine the correlation between drought resistance of KBG cultivars and billbug resistance (as measured by level of billbug damage), we used data from NTEP. The NTEP compiles standardized evaluations of many turfgrass species and hundreds of unique cultivars performed by cooperators across the United States and Canada. Here, we focused on the data

surrounding drought resistance and resistance to billbug damage of KBG, using NTEP data from

trials conducted 1990-2005 (later trials not measuring billbug damage). Billbug resistance is

measured as level of visual damage on a scale from 1 (100% plot damaged) to 9 (no visible

damage), with experimenters confirming billbugs as the cause of damage using the "tug test" and

visual identification of signs unique to billbug damage (i.e. frass within broken turfgrass stems)

(Skogley and Sawyer 1992). Drought resistance is rated both as resistance to wilting (scale 1-9,

9=no wilting) and to dormancy (scale 1-9, 9=no dormancy) (see Morris 2005 for further

descption of turfgrass evaluation methods).

Methods Common to Choice Assays:

Insect Collection

Adult billbugs used in choice assays were field collected using linear and cup pitfall traps

(Dupuy and Ramirez 2016) located at the golf courses previously described. Billbugs were

identified to species and stored in ventilated containers with moistened cotton wicks at 6°C for

no more than one month before use in choice assays. Billbugs were acclimated to room

temperature for 2 hours before use in choice assays.

Plant Material

Four Kentucky bluegrass cultivars —Award, Jump Start, Baron and Midnight— were

selected for use in choice assays (Granite Seed Company, Lehi, UT). These cultivars show a

wide range of drought resistance ratings. Award, considered an "elite" cultivar (Brede 2004),

consistently rates among the best cultivars in terms of visual quality and resistance dormancy

under drought stress (Morris 1995, 2000, 2005, 2011, Bushman et al. 2012, Lewis et al. 2012,

Goldsby et al. 2015) (Table 1). Midnight is considered the standard in Kentucky bluegrass and

has remained popular for many years since its registration in 1984 as a result of its high visual quality and stress tolerance (Bushman et al. 2012). Though it has since been surpassed in drought resistance by a number of cultivars, it still performs well under summer stress and ranks as highly to moderately drought tolerant (Perdomo et al. 1996, Wang and Huang 2004, Richardson et al. 2008, Xu et al. 2011). Jump Start is relatively infrequently studied, but NTEP trials show low dormancy resistance for this cultivar, and for the cultivar Baron (Morris 1995, 2000, 2005, 2011). Specifically, Baron has shown poor visual quality under heat and drought stress in a number of studies (Bonos and Murphy 1999, Lewis et al. 2012, Goldsby et al. 2015). Given the NTEP data and relevant literature, these four cultivars were assigned relative drought resistance rankings (Table 1). Award was ranked as high, Midnight as high-moderate, Jump Start as moderate, and Baron as low. We also compiled NTEP data pertaining to other traits of these cultivars for later analysis (Table 2).

Turfgrass samples for use in choice assays were established from seed under controlled greenhouse conditions. Seeds were sown at the rate of $14.6g/m^2$ into well-drained pots (15.25cm D x 20.32cm H) filled with a 1:3 v/v mixture of Sungro #3 Propagation Mix and sand. The plants were allowed to establish for 30 days in the greenhouse (23°C, 14L:10D, 37% RH) during which time they were watered to saturation every other day. During establishment the plants were fertilized with dilute fertilizer solution (Peter's Excel, B base formulation 21:5:20 NPK) once as soon as shoots appeared (6 days after planting) and every 2 weeks thereafter. Plants were trimmed weekly to maintain 15cm stand height. In 2019 an outbreak of powdery mildew was treated with biorational fungicide (soap) (Mmbaga and Sauvé 2004). All plants used in trials were free of powdery mildew and damage.

Choice Assay 1: Water limitation as a driver of host selection

To test host selection behavior, billbugs were exposed to turfgrass plants using a microcosm choice assay. Here, the experimental unit was a 61cm L x 40.6cm W x 35.2cm H arena (plastic tub) partially filled to a depth of ~20cm with autoclaved sand into which pots containing established turfgrass plants were inserted at opposite ends of the arena, 30 cm apart. One pot contained a well-watered plant, and the other a water-limited plant of the same cultivar. We implemented differential watering treatments, well-watered and water-limited, two weeks before plants were used in choice assays. The soil in pots in the well-watered treatment remained at field capacity (27% VWC) for this treatment period. The soil in pots in the water-limited treatment was allowed to dry down to 4%, then watered and maintained at 17% VWC (60% of the well-watered treatment) for the duration of the two-week treatment period. VWC measurements were taken every two days to maintain plants at their assigned VWC treatment level (FieldScout TDR 100 Soil Moisture Meter, Spectrum Technologies, Aurora, IL). Plant establishment procedure and well-watered/water-limited treatments followed Huang et al. 2011.

Before each trial, the billbug to be used was measured across the widest part of the abdomen using calipers, this measurement serving as a proxy for sex as bluegrass billbugs cannot be reliably sexed visually and response to host VOCs may differ between sexes (Duffy et al. 2018). The billbug was then placed directly between the two equidistant pots, facing perpendicular the plane on which they were aligned. During each trial the billbug was allowed to move freely around the arena. A positive selection of a plant was demarcated when a billbug spent over ten consecutive seconds within approximately 1cm of the edge of the pot, at which point the trial was terminated. This strict metric was used as a marker of choice to reduce the frequency with which the rapid movements of the billbug right after introduction, likely a

response to handling by the experimenter and not indicative of host selection, were marked as an affirmative choice. Furthermore, this selection metric allowed for investigation by the billbug and use of more cue types than VOCs alone, as herbivorous insects also use physical properties (i.e. color and leaf traits) of plants for host selection (Alonso-Pimentel et al. 1998, Awmack and Leather 2002). If this choice did not occur within 10 minutes the outcome of the trial was marked as "no decision" (Duffy et al. 2018). All trials were conducted from late June to early July of 2020, from 16:00MST to 19:00MST. The plants were swapped out for new plants and arena was rotated 180° every 5 trials. The arena's substrate (50L of sand)was mixed after each trial to homogenize any directional billbug cues. Furthermore, after every 5 trials the sand was thoroughly turned over when new holes were created to swap out plants. To create a homogenous visual environment walls of paper were erected around the arena and full spectrum lights were placed over both ends of the arena (OTT-LIGHT, Environmental Concepts, Tampa, FL) (as described in Blackmer et al. 2004). For each of the 4 cultivars (Award, Baron, Jump Start and Midnight), 40 trials were conducted, for a total of 160 trials overall.

Choice Assay 2: Evaluation of Billbug Cultivar Preference

Y-tube assays were used to determine if billbugs discriminate between cultivars of KBG. The four cultivars tested were chosen for their differences in relative drought resistance (Tables 1). The Y-tubes used for these experiments had an inner diameter of 1.5cm, arm length of 7.5cm, and stem length of 8.5cm (The Custom Glass Shop Glass and Plastic Labware, Vineland, NJ) (Fig. 1). Charcoal filtered air was pumped via Buck sampler through a section of Tygon tubing, across the turfgrass sample, and finally through another section of Tygon tubing into each arm of the Y-tube. Similar to Duffy et al. 2018, a flow rate of 1 L/min was maintained in each arm of the Y-tube. Turfgrass samples, consisting of a pot of established turfgrass 15.25 cm in diameter

(see Plant Material section), were contained within plastic enclosure bags (referred to as cue chambers hereafter). The tubing was affixed to the Y-tube using a fitted cork with a muslin covering to prevent billbugs from coming into contact with and leaving odor traces on the cork surface. The tubing sections and cue chambers were assigned to a single cultivar to avoid cross-contamination of chemical cues.

To avoid directional bias the orientation of the arms of the apparatus were reversed every 5 trials, and a full spectrum OTT-LIGHT (Environmental Concepts, Tampa, FL) was placed directly over the arms of the Y-tube such that both were equally lit. A homogeneous visual environment was also created as described in Blackmer et al. 2004 by placing the entire apparatus in a box to exclude visual cues from experimenter movements, and by erecting white paper walls surrounding the Y-tube to obscure air pumps, filters and turf samples from billbugs' view. Glassware was cleaned between cultivar treatments by washing with diluted Liquinox detergent (Alconox Critical Cleaning Supplies, White Plains, NY), first rinsed with acetone, then with hexane, and allowed to dry overnight before use. The plant tissue surface and the pots containing them were carefully rinsed with tap water upon removal from the greenhouse and allowed to acclimate to the new lighting and temperature of the lab for a minimum of 24 hours before use in trials. Three different plants of each cultivar were used in tests, each used in 1/3 (15) of trials for each pairwise comparison where that cultivar was present.

Y-tube trials were conducted between 9:00 and 16:00 hours MST. In each trial a single billbug was measured as previously described and then placed in the base of the stem of the Y-tube which was then blocked such that the weevil could not escape, but air could still pass through the apparatus. Weevils were allowed 10 minutes to make a choice between the two arms of the Y-tube (as in Duffy et al. 2018). A choice was defined as the weevil passing a line marked

1 cm from the branching point of the apparatus. If no choice was made within 10 minutes this was recorded as "no choice".

All plants used in Y-tube assays were from the well-watered treatment (see plant material section above). For each pairwise comparison between our four cultivars, 45 trials were conducted for a total of 6 treatments and 270 trials overall.

Statistical Analysis

Survey 1: Evaluation of adult billbug abundance relative to field conditions

We performed model selection to determine if VWC significantly influenced adult abundance. To determine the relative effect size of changes in VWC compared to other factors —location (golf course, and specific trap therein), soil temperature, and ambient temperature— these terms were also included in model selection procedures. We fit generalized linear mixed models (GLMMs) with negative binomial distribution to control for overdispersion. In all models the golf course where the trap was located was included in the fixed effects terms and the trap number as a random effect to block for repeated collections from each trap, with control for autocorrelated predictor variables. We selected the best-fit model by selecting the GLMM with the lowest Akaike's information criterion (AIC) value. Where models differed by less than 2 AIC units the LMM with the fewest parameters was selected (Burnham and Anderson 2002). All models were fitted using the "glmmTMB" function with autoregressive order-1 to control for structured variance-covariance from the package glmmTMB (version 1.0.2.1) in RStudio (version 1.3.1093). Model fit and residuals were checked using DHARMa (version 0.3.3.0).

Survey 2: Evaluation of billbug damage ratings relative to cultivars with varied drought resistance

To determine the extent to which drought resistance of KBG cultivars correlate with resistance to billbug damage, two generalized linear models (GLMs) with gaussian distribution were created. One model examined the extent to which wilting resistance of KBG cultivars predicted billbug resistance, and the other tested the extent to which dormancy resistance predicted billbug resistance. Twenty-five out of the 433 unique KBG cultivars included in our data were assessed by NTEP trials on more than one year. As a result, each year's assessment was treated as a unique cultivar to account for trials being conducted in different states and by different investigators each year. Considering that the same traits were not always assessed for every cultivar or each year the number of usable datapoints differed for each model (GLM wilting N=346, GLM dormancy N=204). GLMs were run using the stats package (version 3.6.2) in RStudio (version 1.3.1093).

Choice Assay 1: Water limitation as a driver of host selection

For each of the 4 cultivars, 40 trials were conducted for a total of 160 trials overall. To determine if billbugs selected for well-watered and water-limited KBG plants overall, or in any one cultivar we used Chi-square tests for independence (null hypothesis of 50:50 selection ratio). An ANOVA was used to determine if billbug selection of water limited plants differed between cultivars (N=160). We also analyzed the no-response rates, and the time before a decision was made for each cultivar comparison using two ANOVAs (Blackmer et al. 2004). An additional ANOVA was used to determine if, over all cultivars, the size of billbugs choosing each water-limited plants differed from those choosing well-watered (size as a rough proxy for sex, as female tend to be larger). Model assumptions concerning fit and variance of residuals were verified graphically (using DHARMA version 0.3.3.0). Chi-squared tests and GLMs were run using the stats package (version 3.6.2) in RStudio (version 1.3.1093).

Choice Assay 2: Evaluation of Billbug Cultivar Preference

Cultivar-specific chi-squared tests for independence were used to determine if billbug selection rates differed from the expected (null expectation that billbugs would select a cultivar in 50% of trials where it was present). To determine if billbugs selected each of the four cultivars equally (null expectation that 25% billbugs would select each of the four cultivars) an additional Chi-squared test for independence was used. For qualitative analysis, our four KBG cultivars were characterized with regards to billbug damage, overall quality (as described in Morris 2005), drought resistance (dormancy rates), and leaf shear strength using NTEP data compiled from final reports published between 1996 and 2010 as these were the most recent trials to rate billbug damage (Table 2). Mean ratings for each trait were taken from each report to find a single value for every trait for each of the four cultivars. We also analyzed the no-response rates, and the time before a decision was made for each cultivar comparison using two ANOVAs (Blackmer et al. 2004). An additional ANOVA was used to determine whether the size of billbugs choosing each cultivar differed among cultivars (size as a rough proxy for sex, as female tend to be larger). Though we analyzed all 270 trials (6 cultivar comparisons; 45 trials each) to determine response rates, trials in which no choice was made were not included in our primary Chi-squared analyses, so a total of 181 trials remained for use in data analysis. The number of trials for each pairwise comparison of our four cultivars of study was Award*Jump Start=31, Award*Midnight=32, Baron*Award=32, Baron*Jump Start=26, Jump Start*Midnight=33, Midnight*Baron=27. Model assumptions concerning fit and variance of residuals were verified graphically. Chi-squared tests and GLMs were run using the stats package (version 3.6.2) in RStudio (version 1.3.1093).

Results

Survey 1: Evaluation of adult billbug abundance relative to field conditions

During the course of the study, we collected 1072 adult billbugs from our 18 pitfall traps. Collection of each pitfall trap, at each collection date, yielded a single data point for which adult billbug count was associated with our predictor variables (date, location, ambient temperature, soil temperature, and VWC), for a total of 155 data points. Billbug counts showed an extreme left skew, with no billbugs found in the trap in 42.6% of collections, though the maximum collection was 85, and mean was 7.71 ± 1.22. VWC ranged from 6.0 - 71.6, soil temperature from 16-33°C, and ambient temperature from 17-33°C.

Our GLMM model selection showed that location of collection (one of our golf courses) and soil VWC at a trap best predicted the number of adult billbugs collected from that trap (Table 3, Model 1). Our final model contained only these two terms. VWC was the strongest single predictive variable among the abiotic variables we monitored, with significantly more adults captured at traps with lower soil VWC (back-transformed estimate= -3.37 ± 1.02, p=0.011). Increased soil temperature also yielded significantly higher billbug capture when this factor was included along with the terms of our selected model (Table 3, Model 2) (back-transformed estimate= -3.09 ± 0.89, p=0.036). Inclusion of ambient temperature as an additional random effect term decreased the explanatory power of the model in all cases, and was not included in any of the best-fit models (Table 3). Pairwise contrasts of locations of collection (4 different golf courses) showed that all courses were similar in billbugs collected apart from Rose Park which showed the lowest predicted counts (1.08 ± 0.60) and was significantly lower than the course with the highest predicted counts, LCC (6.83 ± 3.48) (p=0.012) and Forest Park (5.27 ±2.67) (p=0.024).

Survey 2: Evaluation of billbug damage ratings relative to cultivars with varied drought resistance

Resistance to wilting (t=6.50, p<0.001) and resistance to dormancy (t=4.27, p<0.001) under drought stress conditions were both negatively associated with billbug damage sustained (estimate = 0.35 ±0.05 and 0.30 ± 0.07 respectively) (Fig. 3).

Choice Assay 1: Water limitation as a driver of host selection

Billbug selection frequency of well-watered versus water-limited plants (58 versus 45) did not provide significant evidence of preference in the 103 (64% of total) trials in which billbugs responded (X^2= 1.64, df=1, p=0.20) (Fig 4A). None of the cultivars showed significant difference in the frequency of plants selected regardless of irrigation treatment, as compared to the expectation of equal selection frequency of the two treatments (X^2=1.84, df=3, p=0.61) (Fig 4B). This was further supported in that the cultivar a billbug was exposed to did not influence the probability that it would select the water-limited plant (F=0.85, df=3, p=0.47). Billbug response rates (F=0.89, df=5, p=0.45) and times (F=0.89, df=5, p=0.49) did not differ between cultivars, and the size of billbug selecting water-limited plants did not differ from those selecting well-watered plants (F=0.096, df=3, p=0.96).

Choice Assay 2: Evaluation of Billbug Cultivar Preference

Billbugs differed in the frequency with which they selected some KBG cultivars in the 181 trials in which they responded (67% of all trials conducted). The moderately drought tolerant cultivar (Jump Start) was selected significantly less frequently (33 times) than the high-moderate tolerance cultivar (Midnight) (55 times) (X^2=5.5, df=1, p = 0.019), though Midnight has demonstrated much greater resistance to billbug damage than Jump Start (Table 2). No other

cultivars showed significant differences in their selection frequency ($X^2 > 0.45$, df=1, p>0.05) (Fig 5A). Comparing within cultivars also showed that the moderately drought tolerant cultivar (Jump Start) was the only cultivar for which selection of it versus non-target cultivars differed significantly from the expected equal ratio (X^2=6.4, df= 1, p<0.05) (Fig 5B). Billbugs' response rates (F=0.56, df=5, p=0.51) and times (F=0.89, df=5, p=0.49) did not differ between cultivars, and the size of billbug selecting different cultivars did not differ (F=0.72, df=5 p=0.61).

Discussion

We found that billbug abundance was greater in areas with lower soil moisture (Fig 2). In fact, VWC was the strongest predictor of the number of billbug adults captured at each trap (Table 3). High billbug abundance in dry areas has the potential to be particularly damaging if the cultivar planted in those areas are also drought susceptible, as billbug damage is higher in drought susceptible KBG cultivars (Fig 3). The combination of drought and billbug stress in such areas could yield unacceptably high levels of damage, or necessitate unnecessarily high rates of irrigation or pesticide use. However, predicting the locations of highest billbug abundance and areas most prone to damage is one way to reduce this need, facilitating more targeted management and use of insecticides. Though adult billbugs are not the damaging phase, research on another weevil pest in turfgrass (the annual bluegrass weevil *Listronotus maculicollis* in *Poa annua*) showed that adult abundance from one year predicted larval damage in the next (McGraw and Koppenhöfer 2009). Thus, predicting adult abundance using VWC may predict larval damage in subsequent years, and provide an alternative to tracking larval abundance which is challenging due to billbug larval development within soil and grass stems. The strength of VWC as a predictor of adult abundance in our model may result from the fact it best captured the

overall character of trap locations. As we used periodic, instantaneous measurements of conditions at our traps, ambient temperature and soil temperature were more prone to fluctuation based on weather at the time of measurement than was VWC, which is slower to change. Nevertheless, our survey suggests that VWC could be a useful tool in predicting locations of billbug outbreaks.

Though billbug adults were more abundant in areas with lower soil moisture, they did not prefer water-limited plants overall in choice assays (Fig 4A). In fact, they did not show a preference for either well-watered or water-limited plants overall. Furthermore, billbugs did not prefer the drought susceptible plants under water-limited conditions, though previous studies have suggested that arthropod herbivores in some systems prefer drought susceptible cultivars under water stress (Showler and Castro 2010, Ruckert et al. 2021). This lack of preference for water-limited or stressed plants suggests that, though billbugs are more abundant in drier areas, adult host selection for water-limited plants does not drive this pattern. Billbug larval survival may play an important role in explaining the abundance pattern we observed. Billbug larval survival is highly dependent upon soil moisture, with mortality rising significantly when greater than 20% of total soil pore space is occupied by water (Reynolds et al. 2016). Thus, wet areas are likely to have lower larval survivorship and would therefore see lower rates of adult emergence. Adults are flightless and limited in their range, so areas with the greatest larval survival are likely to see the greatest adult activity. The pitfall traps that we used to measure abundance rely upon adult activity (movement across the soil surface) for capture, so areas with concentrated emergence would likely also see higher capture rates. For instance, the golf course with the lowest overall adult abundance (Rose Park) suffers severe flooding during late spring each year, likely drowning larvae and precluding any billbug emergence from large areas. On the other

hand, the course with the highest overall adult capture (Forest Dale) has many areas of extremely

rocky, rapidly-draining soil, brought in to create course features and hills. The trap with the

highest cumulative adult capture in our study was found on just such a hillside at Forest Dale.

Evidence of this linkage between adult and larval abundance was also shown in the another

turfgrass-dwelling weevil (*Listronotus maculicollis*) in which cumulative adult capture in a given

location was significantly associated with larval abundance in that location the following year

(McGraw and Koppenhöfer 2010). Though billbug larvae do not choose their hosts, adult

oviposition preferences and subsequent larval survival rates may still drive the correlation

between adult abundance and soil VWC. Study of larval abundance and survival may prove

helpful in explaining the patterns we observed. However, billbug larvae are extremely difficult to

study, so examining adult oviposition preferences could be a simpler way to address the same

questions. Such study is warranted, as female preference for healthy or drought stressed hosts for

oviposition differs between systems (Carr et al. 1998, Showler and Castro 2010), and cannot be

assumed to correlate with conditions best for larvae (Showler and Moran 2003).

   Though drought susceptible cultivars of KBG generally suffered more billbug damage than

did drought resistant cultivars (Fig 3), this does not appear to be a function of billbug adults

selecting drought susceptible cultivars. Billbugs did not prefer more drought susceptible cultivars

in Y-tube assays (Fig 5B), and furthermore did not respond differently toward cultivars of

highest versus lowest drought resistance. Instead, billbugs only differentiated between the two

cultivars central in our drought resistance spectrum, showing a preference for the high-moderate

drought resistance cultivar Midnight over the moderately drought resistant cultivar Jump Start

(p=0.039) (Fig 5A), while all other pairwise comparisons between cultivars showed no

preference. As billbugs do not appear to select drought susceptible cultivars, the increased

damage to drought susceptible cultivars we observed in NTEP trials is not likely a function of

adult selection for those cultivars increasing abundance and thereby increasing damage. Instead,

cross-resistance between drought and billbugs in some KBG cultivars is more likely. There may

be molecular cross resistance to these stressors, with abiotic and biotic stressors inducing

accumulation of phytohormones, transcription factors, kinase cascades, and/or reactive oxygen

species that aid in plant defense (Rejeb et al. 2014). However, correlations between resistance to

drought and resistance to insect feeders can also arise from morphological traits (i.e. leaf traits)

(Saska et al. 2020). Drought resistance can arise via many different plant traits in turfgrass

(DaCosta et al. 2004), and some of these traits (i.e. increased cuticle thickness) may induce

resistance to drought while also increasing resistance to insect damage (Raupp 1985,

Ramegowda et al. 2020). It is also possible that drought resistant KBG cultivars are simply able

to maintain color and vigor under multiple stressors, and often have aggressive growth habits

that would allow them to conceal billbug damage (Kindler and Kinbacher 1975, Lindgren et al.

1981, Ahmad and Funk 1982, Bruneau 1987). While NTEP data did show a negative correlation

between drought resistance and billbug damage, there were many cultivars that showed

idiosyncratic trends (Fig 3). This suggests that a subset of traits associated with drought

tolerance, and not drought tolerance itself, confer resistance to billbug damage. Further

experimentation to investigate cross-resistance between billbug and drought resistance could

pinpoint traits that foster resistance to both drought and billbugs, two key causes of damage to

KBG that could be selected for.

The traits of our study cultivars may provide some suggestion of traits driving billbug

selection and undergirding resistance to billbug damage. The cultivar preferred by billbugs in Y-

tube choice assays, Midnight, has the lowest shear strength among cultivars tested here (Table

2). Soft leaf tissue has been proposed to favor billbugs (Bruneau 1987), potentially due to easier feeding and reduced mandibular wear (Raupp 1985). Despite adult billbug preference for this cultivar and low shear strength, Midnight did not suffer the greatest billbug damage of our test cultivars in NTEP trials, in fact showing damage lower than average (Table 2). It may be that Midnight is able to conceal damage due to its density and vigorous growth habit (Meyer et al. 1984, Bonos et al. 2012). Concealment of damage has been a mechanism proposed for turfgrass resistance to billbugs, with vigorous, healthy cultivars generally showing less damage (Johnson-Cicalese et al. 1989, Shetlar and Andon 2012). Furthermore, Kindler and Kinbacher 1975 found that turfgrasses originating from old fields in regions historically infested by billbugs had higher billbug resistance, and Midnight originated from an old-lawn in Washington DC (Meyer et al. 1984). While Midnight was preferred by adults but was minimally damaged in NTEP trials, Jump Start was least attractive to billbug adults yet suffered the most severe billbug damage of our test cultivars in NTEP trials (Table 2). This discrepancy further refutes the adult host selection as the mechanism driving billbug damage patterns. Other examples of lack of correlation between drought resistance and billbug resistance can also be found in the literature. For instance, while the cultivar 'Kenblue' showed the highest billbug resistance of any test cultivar in Ahmad and Funk 1982, it consistently shows poor drought performance (Keeley 1996). In the same studies 'Merion' suffered severe billbug damage, but was highly drought resistant. From a management perspective, our results suggest that selecting high quality cultivars, not necessarily all drought resistant cultivars, may be the best way to also help prevent billbug resistance. Though drought resistant cultivars tend to suffer less billbug damage, this is not a consistent criterion if the aim is to also select billbug resistant KBG. We now have documentation of multiple cultivars that perform well overall, and show moderate to high billbug

resistance. Such cultivars include: Midnight, with documented billbug resistance and high overall quality ratings (Morris 1995), 'Mystic' with vigorous growth and very strong billbug resistance (Johnson-Cicalese et al. 1989), and cultivars such as 'Plush' and 'Wabash', mid-Atlantic types which show generally strong drought performance (Keeley 1996), and billbug resistance (Johnson-Cicalese et al. 1989).

Turfgrass provides a number of challenges in terms of pest management. Though turfgrass is a system unsurpassed in scale, covering over three times as much land area in the US as any other irrigated crop, levels of acceptable damage are very low compared to large scale crops (Milesi et al. 2005). Acceptable levels of foliage loss range from 20-30% for soybeans, peanuts and sweet potatoes, and cotton growers often accept 15-20% plant mortality due to pests before even beginning to treat for pests (Crow et al. 2021) while premier golf courses are expected to maintain perfect green-cover. Increased frequency and severity of drought, and imposition of irrigation bans pose serious problems for turfgrass managers trying to maintain such standards. Primarily, turfgrass will suffer under these conditions due to water stress if cultivars in use are not adapted for such conditions. Our results show that dry conditions also favor billbug outbreaks, which may lead to additional damage to already water-stressed turfgrasses. High performing, drought resistant cultivars certainly can help alleviate the need for irrigation while maintaining turfgrass quality, while also reducing the likelihood of billbug damage. Such cross-resistance between abiotic and biotic stressors is well documented (Huberty and Denno 2004, Atkinson and Urwin 2012, Ramegowda et al. 2020), but further investigation of the specific traits that induce cross-resistance could help guide cultivar development and selection for billbug and drought resistance, two serious problems in the West in particular.

The findings of our survey and cultivar analysis showed high billbug abundance in drier turfgrass areas and more severe damage to drought susceptible KBG cultivars. However, we did not find adult host preferences for water-limited or drought susceptible KBG that might have explained different levels of abundance and damage. Future research should examine other potential mechanisms for resistance, to aid in billbug management. In the short term, our research suggests that turf managers may target monitoring and chemical management to areas they known to maintain low soil moisture throughout the year. Selection of high-quality, drought tolerant cultivars may also reduce the chance of severe billbug damage. There is a need to update studies conducted concerning KBG cultivars and billbug resistance, as the primary studies on this topic were conducted over 30 years ago (Kindler and Kinbacher 1975, Lindgren et al. 1981, Ahmad and Funk 1982, Johnson-Cicalese et al. 1989), and influx of new KBG germplasm renders many of the cultivar recommendations out-of-date. Future research could help refine cultivar selection criterion for simultaneous drought and billbug management by pinpointing modern cultivars that show the greatest cross resistance, and determine what traits are responsible for cross tolerance to guide cultivar development.

Acknowledgements

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Cultivar	Mean NTEP Drought Resistance	Published observations	Sources
Award	5.5	Best ability of cultivars tested here maintain green cover and recuperate after extended water limitation[1] Ranked extremely high for maintaining green color under deficit/no irrigation[1,2,3] Considered an "elite" cultivar in terms of performance, consistently ranking high in quality [5,6]	1. (Goldsby et al. 2015) 2. (Lewis et al. 2012) 3. (Bushman et al. 2012) 4. (Bonos and Murphy 1999) 5. (Morris 1995, 2000, 2005, 2011) 6. (Brede 2004)
Baron	4.3	On average, fastest decrease in percent green cover under zero-irrigation conditions, compared to other cultivars tested here[1] Highly variable in ability to maintain green cover and recover after water-stress[1] Exhibits poor growth and performance under summer stress[4]	
Jump Start	4.5	Average drought resistance (dormancy resistance) similar to Baron[5] Better resistance to wilting than Baron[5]	
Midnight	5.1	Considered an industry standard for high performing KBG3, consistently ranked among highest in tested cultivars in terms of quality[5] Consistently ranks high in maintenance of green cover under deficit/no irrigation[1,2,3] and resistance to dormancy[5]	

Table 1 Drought resistance ratings and characteristics of cultivars of study. NTEP rating for

drought resistance calculated as the mean dormancy score from the 1991-95, 1996-2000, and

2001-05 National Kentucky bluegrass test final reports. Dormancy was visually rated as a

percentage, with 9=0% dormant and 1=100% dormant after a period of zero-irrigation. Overall

NTEP mean for drought resistance (dormancy) was calculated as 4.8, across all 3 study periods

(1991-95, 1996-2000, and 2001-05) and all 433 unique cultivars from 1991-2005.

	Billbug Resistance	Dormancy Resistance	Overall Quality	Shear Strength (NM)
Award	7.33 ± 1.00	5.45 ± 0.65	6.47 ± 0.25	53
Baron	7.1 ± 1.37	4.25 ± 0.25	5.97 ± 0.17	54.8
Jump Start	5.2 ± 0	4.5 ± 0	5.7 ± 0	NA
Midnight	6.9 ± 1.66	5.1 ± 1.00	6.53 ± 0.25	49
Test Cultivar Average	6.63	4.83	6.17	52.27
All NTEP Cultivar Mean	6.68	4.76	5.94	52.8

Table 2 Mean ± std dev values for billbug damage rating (0-9; 9=no damage), mean dormancy (0-9; 9=no dormancy), mean quality (0-9; 9=highest quality; a combination of color, density, uniformity, texture, and disease or environmental stress) rated visually in NTEP trials. Mean ratings for each trait for each cultivar were calculated from data from three final reports encompassing all trials conducted between 1996-2010, with each cultivar having one rating in each report except for Jump Start which was only in the 2005 report. Shear strength measured only in NTEP 2000. See (Morris 2005) for experimental details. Test cultivar mean calculated as mean of only 4 test cultivars. All NTEP cultivar means show the mean rating for all cultivars tested in NTEP trials for that traits from 1996-2010.

	Random Effects	AIC
1	**Location + VWC**	**753.6**
2	Location + VWC + Soil Temp	755.0
3	Location + Soil Temp	758.4
4	Location	759.4

Table 3 The best-fit models in GLMM model selection predicting the number of adult billbugs collected at pitfall traps based on traits of that trap: golf course where trap was located (location), volumetric water content of soil at that trap (VWC), temperature of the soil at that trap (Soil Temp) and ambient temperature at that trap (Ambient Temp). Bolded model was selected based on AIC criterion.
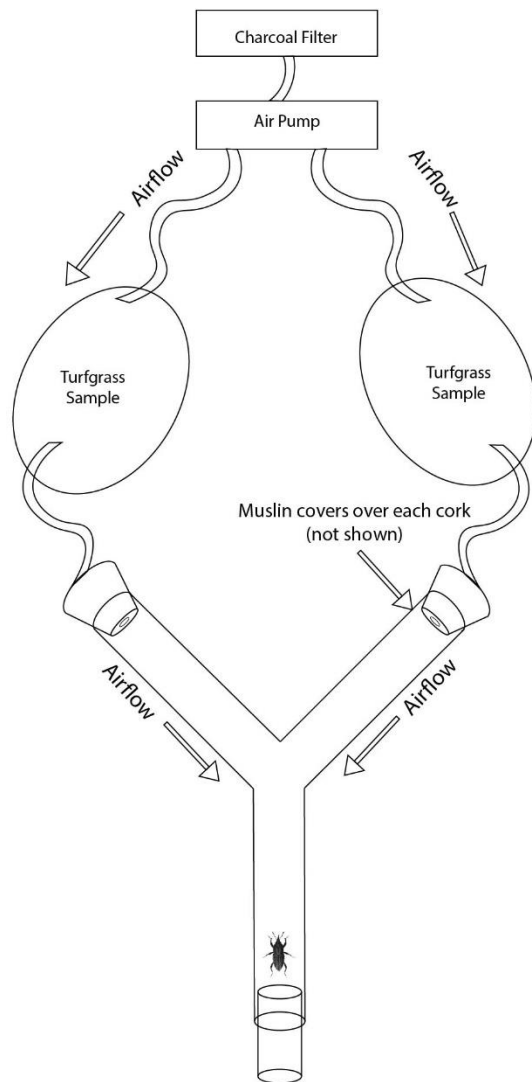
Fig. 1 Y-tube apparatus used in assays had an inner diameter of 1.5cm, arm length of 7.5cm, and stem length of 8.5cm. Charcoal filtered air was pumped into the apparatus (top), through Tygon tubes, into the cue chambers containing turfgrass samples, and finally into the arms of Y-tube. For each trial a single billbug was placed in the base of the Y-tube and allowed 10 minutes to select one of the arms.
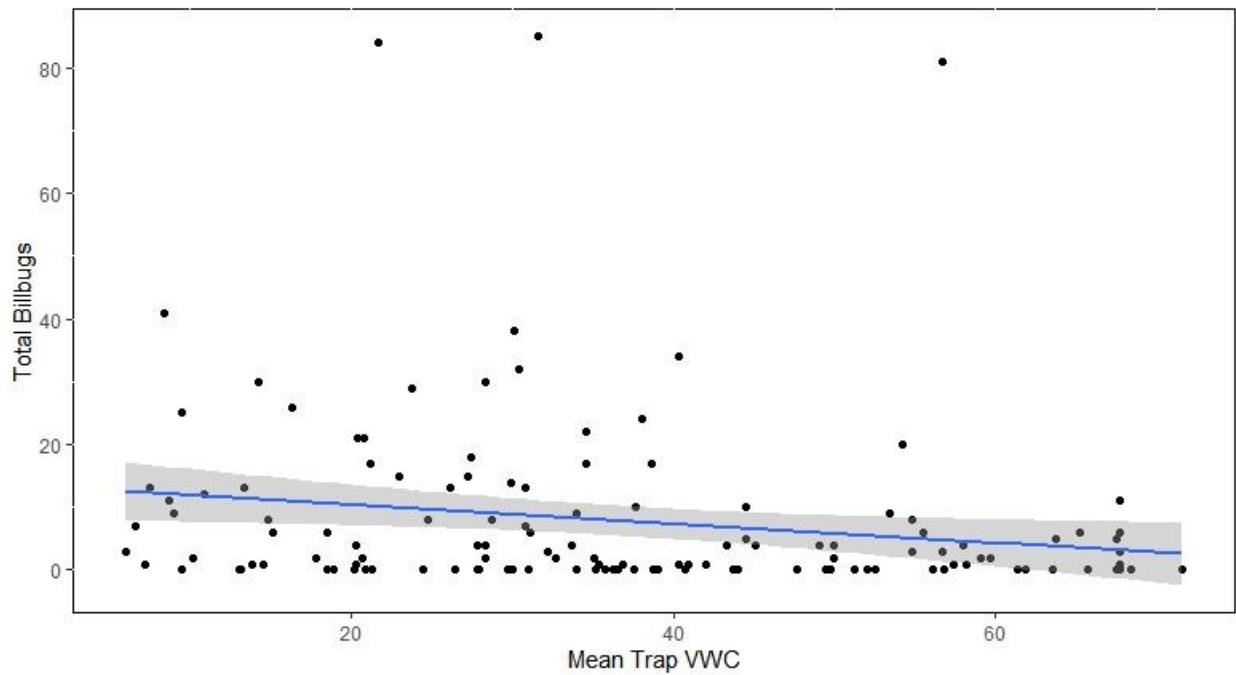
Fig. 2 Each point represents the number of billbugs collected at a single collection at one of 18 pitfall traps. Traps were collected weekly or biweekly over the course of the 2019 and 2020 field seasons. Volumetric water content (VWC) of the soil surrounding the trap was measured at each collection (N=155). Increased VWC at a trap significantly decreased number of adult billbugs collected from that trap (z=-2.55, p=0.011).
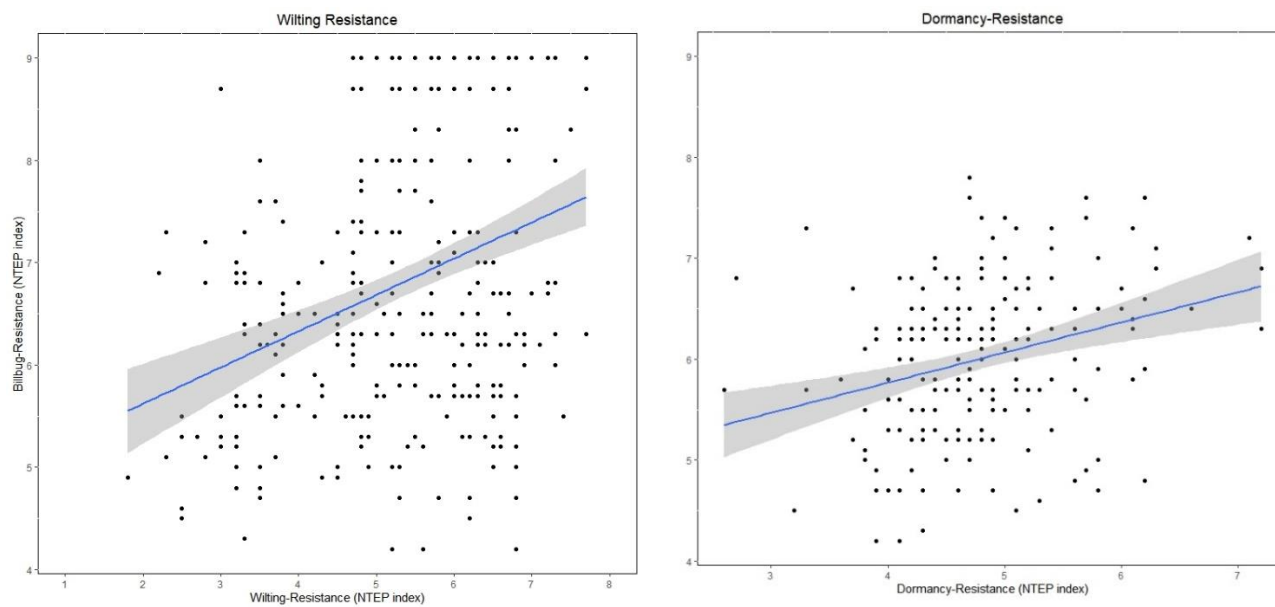
Fig. 3 The level of billbug damage of KBG cultivars was lower in cultivars with greater wilting resistance than those more prone to wilting under drought stress conditions (t=6.50, p<0.001, estimate = 0.35 ±0.05) (A). Billbug damage sustained by cultivars resistant to dormancy was also lower than those more susceptible to dormancy under drought stress conditions (t=4.27, p<0.001, estimate = 0.30 ± 0.07) (B). Wilting and dormancy resistance were measured under zero-irrigation conditions and is given as an index 1-9 (9=no wilting/dormancy). Resistance to billbugs was visually assessed as a proportion of plot with damage on a scale from 1-9 (9=no damage) ($N_{wilting}$ = 346 $N_{dormancy}$=204).
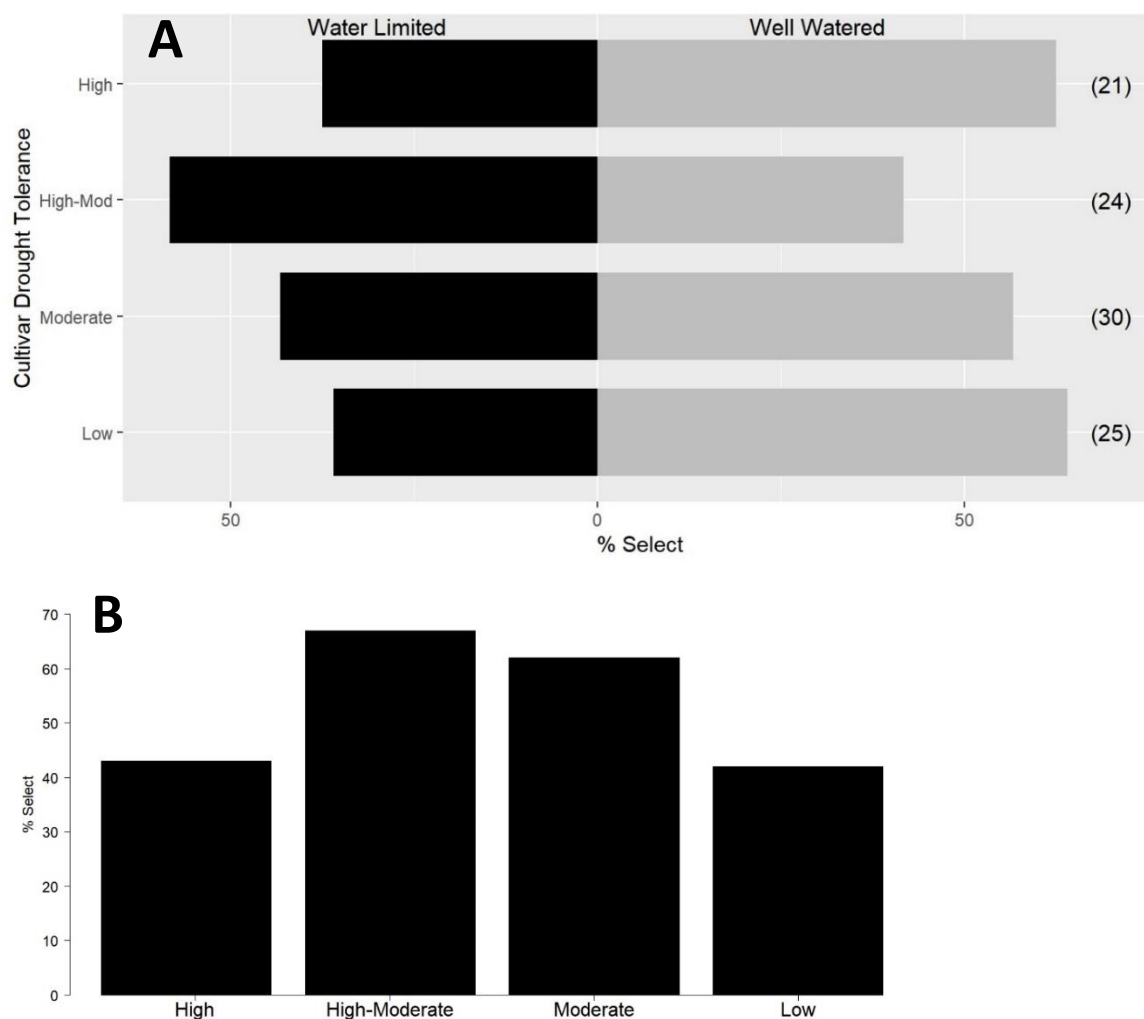
Fig. 4 (A) Proportion of trials where water-limited plant (left bars) and well-watered plant (right bars) of each cultivar, arranged vertically by drought resistance, were selected. Numbers within parentheses show sample size for each cultivar. Cultivars are arranged vertically by drought resistance. No differences were found in selection of well-watered versus water-limited cultivars individually or overall (58 versus 45) (X^2= 1.64, df=1, p=0.20) (N=45). **(B)** No between-cultivar difference was found in selection frequency of water-limited plants of different cultivars, arranged in order of drought resistance from left to right (X^2=1.84, df=3, p=0.61). Billbugs that did not measurably respond were not included in analyses, leading to slightly different sample sizes for different cultivars (N=103).
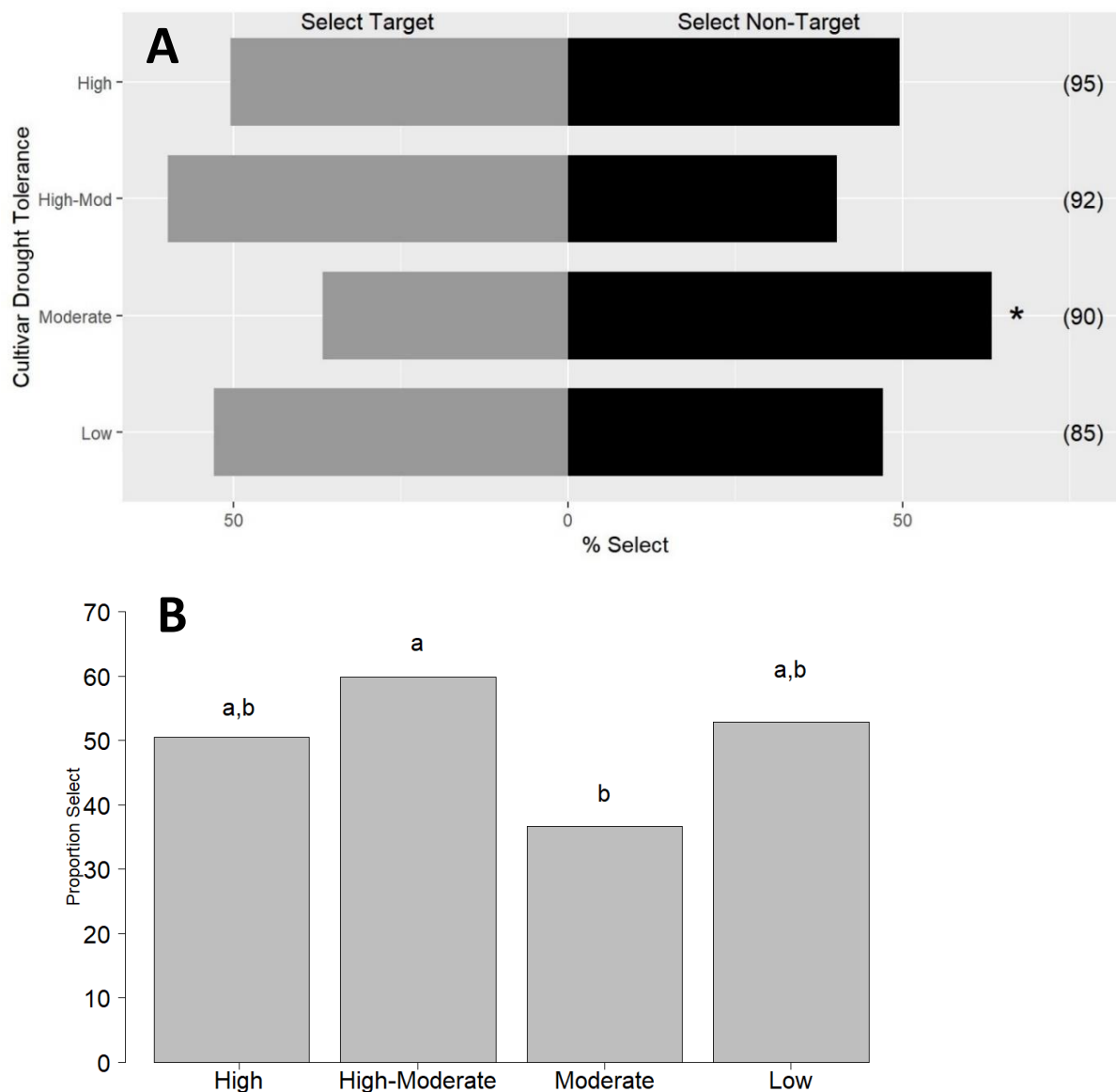
Fig. 5 (A) Proportion billbugs selecting target cultivar listed on y axis (right bars) versus selecting other, non-target cultivar (left bar) in Y-tube choice assay. Numbers within parentheses show sample size for each cultivar. Cultivars are arranged vertically by drought resistance. Significant X^2 (p <0.05) indicated with *. Billbugs that did not measurably respond were not included in analyses, leading to slightly different sample sizes for different cultivars (N=181).

(B) Frequency of selection of KBG cultivars arranged in order of drought resistance, left to right. Significant difference between cultivars (X^2 p<0.05) indicated by different letters (N=181).

CHAPTER IV

GENERAL SUMMARY AND CONCLUSIONS

General summary

Current billbug management options are extremely limited, relying heavily on preventive applications of systemic, long-residual insecticides. There is an increasing need for the development of alternatives to assist in the management of this challenging pest. Furthermore, in the water-limited western United States in particular, there is a need to reduce the input of water to turfgrass systems, while still maintaining functional and aesthetic quality. Fortunately, there are multiple lines of billbug research that are building the foundation of sustainable management of this pest. Here, I add to that foundation with research that can assist in the development of both biological and cultural control strategies.

In my first study, I examined the influence of predator presence and different predator cues on billbug behavior. I used microcosm and Y-tube choice assays to document behavior, and isolate different cues that may facilitate billbugs' detection of predators. I found that billbugs respond to the presence of predators with reduced feeding and mating, and increased predator avoidance behavior. They also show similar responses to predator odor, and avoid predator odor in choice assays, suggesting that chemical signaling is a part of billbug predator detection.

In my second study, I examine the interactions between billbugs and Kentucky bluegrass hosts with varying levels of irrigation and drought resistance. I conducted a golf-course survey, documenting adult billbug abundance in relation to soil moisture. I also analyzed data on Kentucky bluegrass cultivars to examine the correlation between drought resistance and billbug

resistance. I then used microcosm and Y-tube choice assays to determine if billbugs prefer water-limited or drought susceptible hosts. Survey data showed greater adult billbug abundance in areas with lower soil moisture, and that billbug damage was more severe in drought susceptible Kentucky bluegrass cultivars. Though billbugs did show preferences among test cultivars, there was no preference by adult billbugs for water-limited or drought-susceptible plants.

Conclusions

Management of billbugs in turfgrass provides a number of challenges. Many common insecticides used in turfgrass are seeing increased restrictions on their use, so there is a need to reduce our reliance upon chemical control techniques. Limited water resources are also being diverted away from turfgrass irrigation in many areas, so finding ways to reduce irrigation while maintaining turfgrass quality is also a critical need in the turfgrass industry. Billbugs are a pest in turfgrass for which few non-chemical control tactics exist, and which disproportionately impact drought-stressed turf. My research here addresses both of these problematic traits of billbugs in turfgrass, attempting to find ways to manage billbug populations without relying on heavy chemical and water use. My findings show promising results for the implementation of both biological and cultural control of billbugs in turfgrass. I demonstrate that resident predators in turfgrass may contribute to billbug suppression, and discover that billbugs respond to predator odors. I find that billbug abundance and damage are influenced by soil moisture and turfgrass drought-susceptibility. Though differences in abundance and damage are not due to adult host preferences, billbugs do show an ability to differentiate between turfgrass cultivars. These results provide short term solutions to billbug management, showing the merit of conservation biocontrol and cultivar selection. Furthermore, this research provides the platform for additional

research on billbugs biological and cultural control, using findings presented here concerning the

nature of billbug predator detection and host selection tendencies.

APPENDICES

APPENDIX I

SUPPLEMENTARY DATA

1. Methods

The experimental design used here (referred to as the 2018 study) mirrored those used for behavioral assays presented in Chapter II (referred to as the 2019 study), with a few key differences. Insect collection differed in that billbugs, predators, and non-predatory arthropods were only collected from a subset of courses used for the 2019 study: Logan Country Club, and Utah State University's Greenville Research Farm, and greenhouses (41.7661, -111.8107). The same two-chamber experimental arena (Chapter II Fig 1), and method of behavioral observation and data collection was used in both years. Some behavioral definitions were refined from 2018 (Appendix I, Table S1) to 2019 (Chapter II Table 1). The most important methodological difference was that while in the 2019 study four bluegrass billbugs (*Sphenophorus parvulus*) (Coleoptera: Dryophthoridae) were used in each trial, in the 2018 study presented here two bluegrass (unidentified sex) and two hunting billbugs (*S. venatus*) (mating pair) were used in each replicate. All treatments and replicate numbers remained the same other than the non-predatory arthropod treatment, for which only one taxa was used (*Armadillidae* sp.) (n=4). Data analyses performed followed those presented in Chapter II, with GLMs comparing different behaviors between different predator number treatments, and different predator cue treatments.

2. Results

Only one billbug of the 416 observed was consumed by predators in this assay (0.2% consumption), with 7 attacks on billbugs (1% of observations in the direct predator contact condition). Mortality and attacks all resulted from *Pterostichus melanarius.* Despite low rates of

consumption and attack by predators, feeding frequency decreased in all treatments compared to the control ([z]>2.87, p<0.05), with exception of the indirect predator contact treatment which did not differ (z= -2.01, p=0.23) (Supp. Fig. 1A).

The mean frequency of billbugs' predator avoidance behaviors did not differ significantly between predator or predator cue treatments ([z]<1.46, p>0.05) (Supp. Fig 1B).

Billbug mating frequency was extremely low in assays, only observed in 26 of 728 (4%) of observations (Supp. Fig 1C). No treatments significantly differed from the no predator control (0 mating events) (z=1.00, p=1.00). The treatment with the highest mean mating frequency observed was the non-predatory arthropod treatment (1.5 ± 1.5). The non-predatory arthropod treatment differed only from the indirect predator contact treatment (z=3.22, p=0.0081) and the direct predator contact treatment (z=4.20, p<0.001). All other treatments were statistically similar ([z]<1.90, p>0.05).

Billbugs in direct contact with two predators showed significant decreases in feeding (z= -6.02, p<0.001), and marginally significant decreases in feeding when in indirect contact with two predators (z= -2.32, p=0.053). When in direct (z= -2.17, p=0.077) or indirect (z= -1.20, p=0.45) contact with a single predator species billbugs did not feed less frequently (Supp. Fig 2A & 2C). We found no significant differences in the frequency of predator avoidance behaviors between the 0,1 and 2 predator treatments when billbugs were in direct contact (Supp. Fig 2B) or indirect contact (Supp. Fig 2D) with predators ([z]<1.00, p>0.5).

3. Discussion

Despite the fact that this 2018 study examined the behavior of two billbugs species together, it still showed patterns of behavior very similar to those observed in the final 2019

study (Chapter II) in which only bluegrass billbug was observed. We found significant decreases in feeding frequency in response to predators and their cues, and observed general trends toward increased predator avoidance and decreased mating that prompted further replication. Evidence of billbugs using chemical cues to detect predator presence, and surprising alterations in billbug behavior in the presence of non-predatory arthropods prompted follow-up with Y-tube choice assays and further replication of the non-predatory arthropod treatment with an additional species in 2019. We also found that exposure to predator pairs lead to significant decreases in billbug feeding compared to both the no predator control and to single predator exposure (Supp Fig 2A & 2C), a result largely corroborated in 2019 (Chapter II Fig 4). Though increases in predator avoidance were not significant, mean avoidance tended to be higher when exposed to predator pairs, as opposed to no predators or a single species (Supp Fig 2C & 2D). We explored these patterns more fully in 2019, with many of the same general findings (Chapter II Fig 4).

It is noteworthy that we found many of the same responses in this experiment when two species of billbugs present, as when only bluegrass billbugs were present in our follow-up in 2019. Prior work had examined only single billbug species responses (Dupuy and Ramirez, 2019), and our primary experiment in Chapter II isolated billbug species as well. However, in the field billbugs exist in species complexes. As interspecific interactions alter NCEs, the influence of multi-species interactions is key to understanding how NCEs observed in the lab will scale up in the field.

Though this design, using both bluegrass and hunting billbugs, was designed to more fully represent the species complex of the Intermountain west, with these two species being the primary constituents (Dupuy and Ramirez, 2016), this confounded our results. We could not reliably identify species during the assays, particularly when billbugs partially buried

themselves, and so could not differentiate between behavior of the two billbug species. This meant that we were unable to determine if patterns were disproportionately driven by species-specific responses, or if results were common between the two. Furthermore, interaction with both conspecifics and heterospecifics alter risk-induced behavioral responses in insects (Ingerslew and Finke, 2018). As one of our primary objectives was to determine which cues are used by billbugs to detect predators, we altered the experimental design to include only bluegrass billbugs to reduce any potential confounding interactions.

This study allowed us to refine observational and experimental technique in ways that benefited the final 2019 study. Foremost, we were surprised by the behavioral responses observed when billbugs were in indirect contact with a non-predatory arthropod. We suspected that these anomalous results were an artifact of conducting these trials last, as this treatment was only added to the design after the others had been conducted. Being last, the trials of the non-predatory arthropod treatment were conducted with some of the last remaining billbugs from collection that had been stored in refrigerated conditions for over a month. This led us to include another non-predatory arthropod species in the 2019 trials, to ensure the isopod did not have unique effects. It also highlighted the need for randomization of the order in which trials were run, something we did for the assays in 2019. We also refined our behavior matrix from 2018 (Supp Table S1) to 2019 (Chapter II Table 1). In this study we discovered behaviors not yet documented in the literature, some of which proved difficult to see and differentiate. Most notably, billbugs tendency to partially bury themselves in the soil had never been documented outside overwintering adults, to our extent of knowledge. Partial burial almost always occurred at the base of grass stems, so determining when the head was near the base of grass for feeding versus when the billbug was beginning to bury itself necessitated refining our definition. To do

so we added the stipulation that only when the rostrum was observed to be penetrating inside the turfgrass plant was the billbugs listed as "feeding".

Overall, these data show patterns matching those observed in our final, 2019 study (Chapter II). Though we improved some elements of the experimental design, benefiting the final study, we found highly similar results across years. The aim of this work was to provide actionable evidence of billbug NCEs and information on what cues they use to detect predators, so the generalizability of our results across billbug species, and the ecological validity of our findings are key. The similarity in these data compared to those presented in Chapter II suggests that behavioral changes in the presence of predators will hold across billbugs species, and when billbugs are interacting in their natural species complexes.

Table 1. Observed behaviors in billbugs and their descriptions.

| Global Behavior	Specific Behavior	Behavior Description
Mating	Mating	Active entry of aedeagus into female terminalia
Feeding	Feeding above crown	Active feeding upon the stolon, stem, or blade of grass.
	Feeding at base	Active feeding upon the crown of the plant, or the tissue of the plant directly at/below ground level.
Predator Avoidance	Head in soil	Billbug observed to have at least the entire head capsule, and up to the entire body, below the soil surface. Almost always the billbug was oriented vertically, with head facing downward, when this behavior was recorded.
	Crown	Billbug residing, motionless, on any plant structure above the soil surface, and above the crown of the plant, such as upon the stem, or at the apex of the blade.
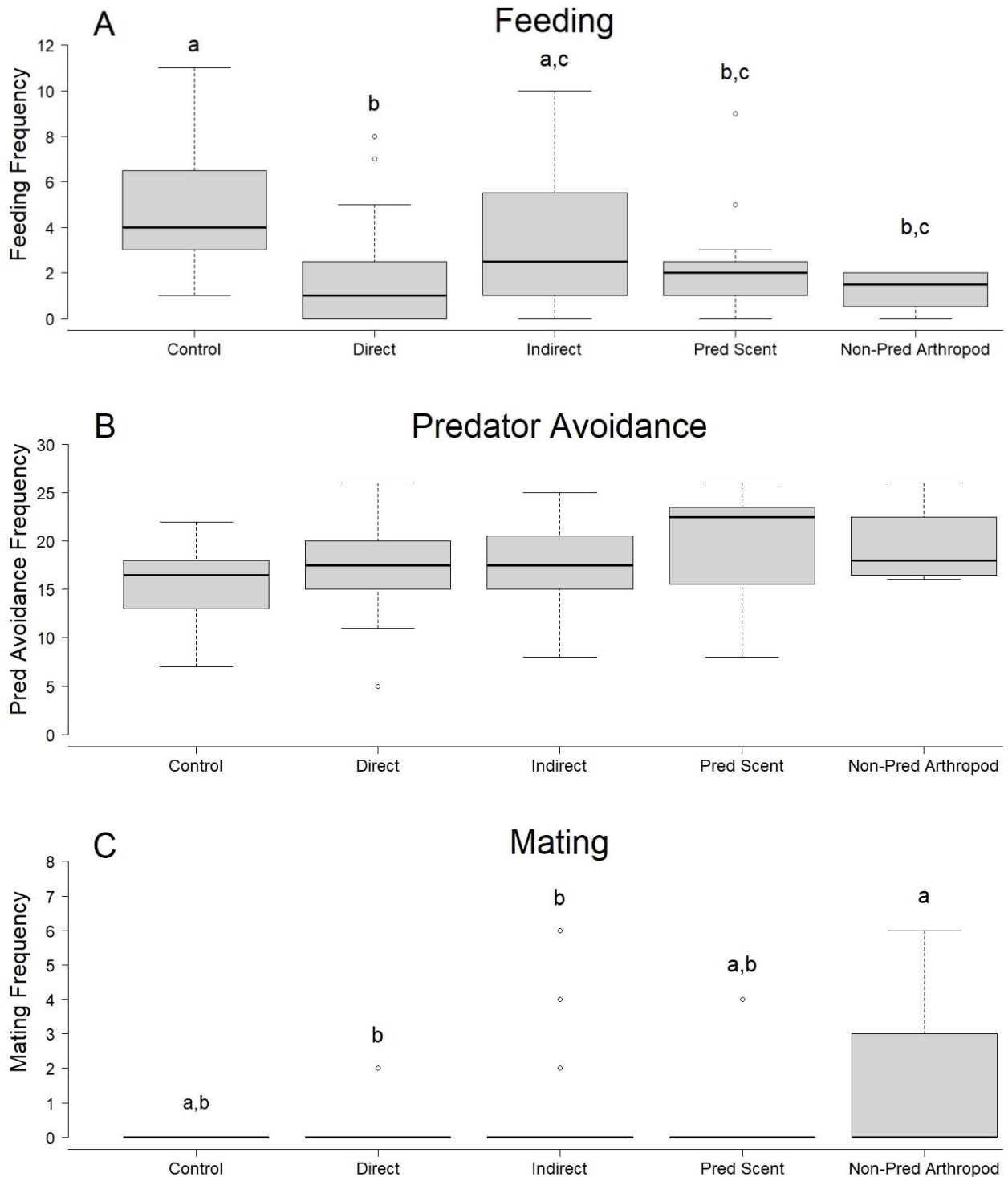
Figure 1: Frequency of observed feeding behavior (A), predator avoidance behavior (B), and mating (C) by billbugs per replicate (two- chamber arena) while billbugs were in control, non-predatory arthropod, predator odor, indirect and direct contact conditions. Conditions assigned different letters indicate significantly different behavioral frequency between conditions (p< 0.01) N=104.
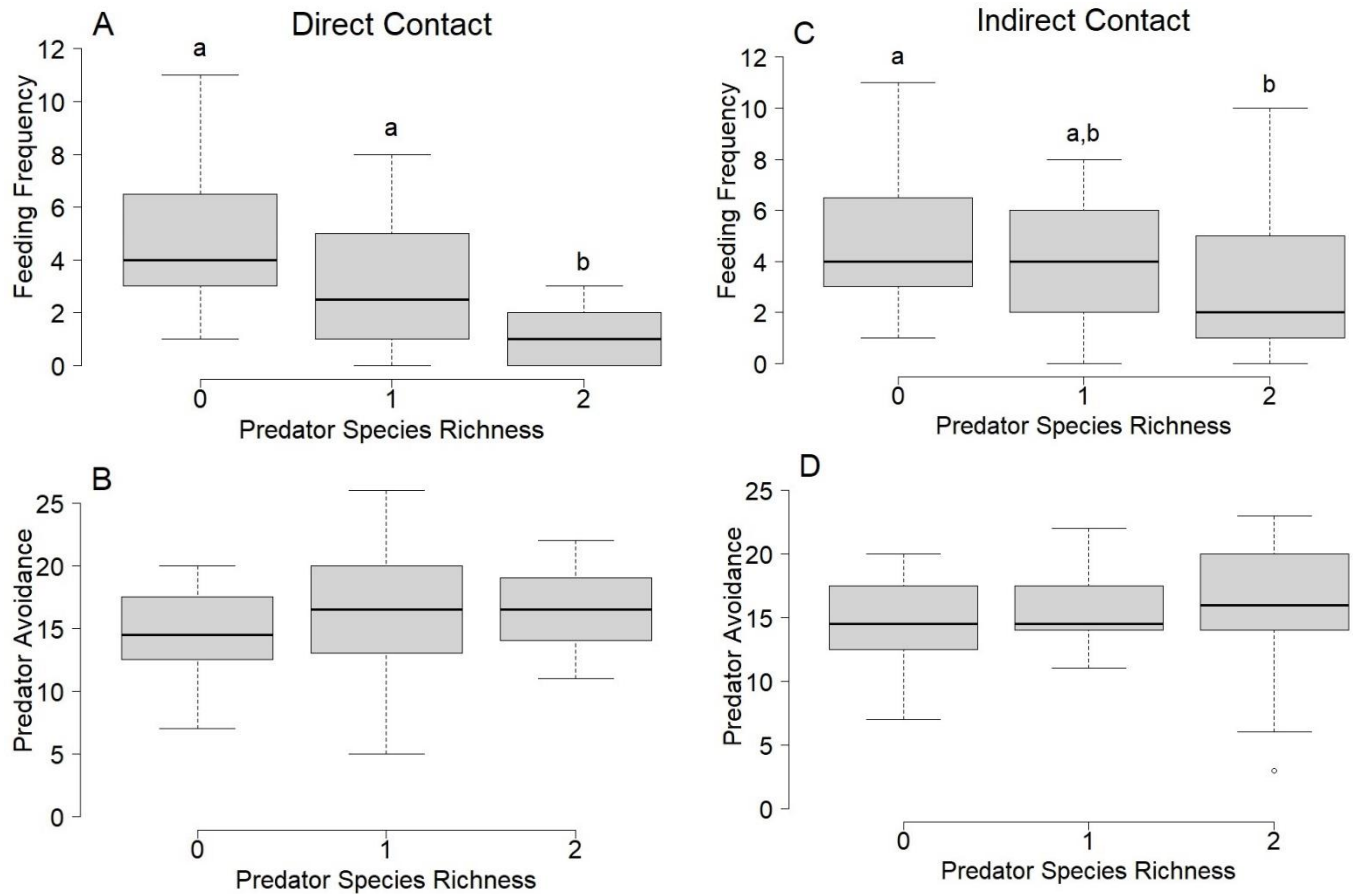
Figure 2: Count of billbug feeding (A & C) and predator avoidance (B & D) observed in each replicate (2 chamber arena) where billbugs were in direct contact (left) (n=48) and indirect contact (right) (n=48) with 0, 1, or 2 species of predators (n=8, 16, 24 respectively). Different lowercase letters indicate significantly different behavioral frequency between conditions (p< 0.05).