

THE PHYSIOLOGICAL AND BEHAVIORAL RESPONSES OF THE  
LESSER CHESTNUT WEEVIL, *CURCULIO SAYI* (GYLLENHAL), TO  
POTENTIAL ATTRACTANTS: DOSE-RESPONSE AND  
INTERACTIONS AMONG HOST PLANT VOLATILE ORGANIC  
COMPOUNDS

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By  
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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled:

THE PHYSIOLOGICAL AND BEHAVIORAL RESPONSES OF THE LESSER  
CHESTNUT WEEVIL, *CURCULIO SAYI* (GYLLENHAL), TO POTENTIAL  
ATTRACTANTS: DOSE-RESPONSE AND INTERACTIONS AMONG HOST  
PLANT VOLATILE ORGANIC COMPOUNDS

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## ABSTRACT

### THE PHYSIOLOGICAL AND BEHAVIORAL RESPONSES OF THE LESSER CHESTNUT WEEVIL, *CURCULIO SAYI* (GYLLENHAL), TO POTENTIAL ATTRACTANTS: DOSE-RESPONSE AND INTERACTIONS AMONG HOST PLANT VOLATILE ORGANIC COMPOUNDS

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The lesser chestnut weevil, *Curculio sayi* (Gyllenhal), is a native pest that can infest over 90% of a chestnut harvest if not properly managed (Brooks and Cotton 1929). Current management techniques consist of pesticide applications, and in extreme cases, a postharvest treatment of nuts (Payne et al. 1983, Hunt et al. 2009). More recent work, however, has identified eight volatile organic components of chestnut odors as potential attractants for use in monitoring traps (Keesey 2011). (E)-2-hexenol, 2-heptanol, (E)-2-hexenal, 2-heptanone, ethyl butyrate, ethyl-2-methyl butyrate, ethyl tiglate, and ethyl isobutyrate were tested at four different doses to determine at what concentration they were physiologically detectable and behaviorally attractive. At the highest dose (E)-2-hexenol, (E)-2-hexenal, 2-heptanone, and ethyl butyrate received the most promising behavioral and physiological responses and were therefore selected for further study. Testing compounds individually, however, did not result in any compounds that were clearly attractive behaviorally; so synergistic effects were examined between compounds by testing compound mixtures. Mixtures were more attractive to adult *C. sayi* than individual compounds, and the highest behaviorally response was to the binary mixture of (E)-2-hexenal and 2-heptanone.

**CHAPTER I:**  
**LITERATURE REVIEW**  
**Chestnut Natural History**

**Chestnut Phylogeny**

Chestnut tree phylogeny is complex with multiple species that have varying distributions in the northern hemisphere. The genus *Castanea* within the family *Fagaceae* contains species including the American chestnut (*C. dentata*), the Chinese chestnut (*C. mollissima*), the European chestnut (*C. sativa*), and the Japanese chestnut (*C. crenata*) (Lang et al. 2006). *Castanea* is monophyletic with *C. crenata* as the basal species and two sister clades, one with the three Asian species and the other with the American and European species (Lang et al. 2007). Recent evidence suggests that the genus originated in eastern Asia diversifying within the continent before dispersing to Europe and North America in the middle Eocene and finally diverging further between Europe and North America in the late Eocene epoch (Lang et al. 2007). The North American species make up a clade with the Ozark chinkapin (*C. pumila* var. *ozarkensis*) as the basal lineage that is sister to the Allegheny chinkapin (*C. pumila* var. *pumila*) and American chestnut (*C. dentata*) (Lang et al. 2007). Molecular results also suggest that the morphological trait of a single nut per bur present in species on different continents may have evolved separately (Lang et al. 2006, 2007).

**American Chestnut**

The American chestnut is a historical fixture in the American environmental imagination that has recently faded in popularity. The distribution of American chestnuts across the eastern United States and concentrated among the Appalachians, made it an

economically critical tree for Americans before the 1900's. American chestnuts were used in a wide variety of ways including timber production, nut production, and for tanning processes (Anagnostakis 1987, Barakat et al. 2009). Rural communities especially relied on chestnuts, with some mountain communities going so far as using nuts as currency. The rural and poor demographics of chestnut consumption before the major decline has left folklore as the primary source of information, perhaps best exemplified by the image of roasted chestnuts during the holidays. The American chestnut made up nearly 50% of Appalachian ecosystems in some areas during the 19th and early 20th centuries (Russell 1987). However, the prominence of American chestnut began a swift decline in 1905 when the first case of chestnut blight was reported (Merkel 1905), resulting in 3.6 million hectares of dead or dying American chestnuts within 50 years (Anagnostakis 1987, Russell 1987). Although the infection spread through nearly the entire American chestnut range the blight did not kill isolated patches, the oldest and largest trees, or the youngest trees. Currently this has left American chestnuts as largely an understory tree growing for about 7 or 8 years before they succumb to the blight or stumps sprouting only to die after becoming susceptible to the blight.

### **Chestnut Blight**

The chestnut blight is caused by the fungal pathogen, *Cryphonectria parasitica*, which directly attacks chestnuts by girdling them, which indirectly makes the trees more vulnerable to other damaging influences (Griffin 2000, Hillman and Suzuki 2004). In 1905 when the blight was first officially recognized in the United States at the New York Bronx Zoo (after being imported from Asia) the problem was localized to New York State (Merkel 1905). Control and containment was attempted using a pruning and



spraying program with Bordeaux mixture but it failed and the blight became an epidemic (Murrill 1906). *Cryphonectria parasitica* spread through the native range of the American chestnut and although several notices went out about local control the speed of the outbreak made it devastating (Murrill 1906, Baxter and Gill 1931). The pathogen followed a similar pattern in Europe when it was found first in Italy in 1938 and then it quickly spread through the range of the European chestnut (Woodruff 1946). Comparisons of the spread of *C. parasitica* between the two regions and closely related species of chestnut suggest that the European chestnut is more resistant than the American chestnut (Hebard 1982, Anagnostakis 1987). Aside from innate resistance another factor has allowed both European and American chestnuts to survive the blight, that of hypovirulence. Initially, simply adding a hypovirulent or less deadly strain of *Cryphonectria parasitica* may seem like more of a bad thing, but hypovirulent strains don't just compete for the tree's dying resources they actually interfere with virulent strains. Hypovirulent strains have been used to treat infected trees by applying them to the cankers caused by virulent strains converting and reducing their virulence to ultimately allow the trees to survive. This does not prevent further infection and damage caused by untreated future cankers, but the presence of the hypovirulent strain may give protection to the trees at the most vulnerable points in their life cycle. Unfortunately there is not a single hypovirulent strain that can be inoculated into all the chestnuts infected (Anagnostakis 1987). Rather there are different strains with differing levels of compatibility. It follows that in environments with more diversity of virulent strains, like the Americas, hypovirulent conversion is less effective due to higher levels of incompatibility. Europe, however, has a lower level of *C. parasitica* strain diversity and

so both direct treatment of cankers along with the natural spread of hypovirulent strains has more effectively controlled the damage (Anagnostakis 1987). Hypovirulence and innate resistance are useful ways to manage chestnut blight but don't deal with the origin of the pathogen. Since *C. parasitica* is an invasive pathogen the question must be asked, "How do Asian chestnuts deal with the infection and can similar methods be applied to American and European chestnuts?"

### **Asian Chestnuts and Innate Blight Resistance**

*Castanea mollissima* and *Castanea crenata*, the Chinese and Japanese chestnuts, respectively, both have innate resistance to *C. parasitica* that leads to slower canker development (Anagnostakis 1987, Barakat et al. 2009). Attempts to introduce these desirable traits to American and European chestnuts have become both more complex and effective. Initially attempts were made to simply cross Asian and American chestnuts but the results were often hybrids not blight resistant enough or not viable forest trees (Anagnostakis 1987). The issue is that blight resistance is not the only trait being transferred or disrupted in simple crosses. Cold resistance is may be the biggest problem but American chestnuts have also become environmentally adapted in many other ways (Anagnostakis 1987). For this reason even from an economic perspective simply replacing American chestnuts with Asian varieties is not a viable option in many areas. Although Asian chestnut varieties are successful in some areas (Hunt et al. 2009), the conservation goal remains to retain all American chestnut genes while only including blight resistance. The American Chestnut Foundation has pursued this goal by aggressively backcrossing resistant hybrids with American chestnuts repeatedly ("Restoring the American Chestnut" 2007). Besides approaching the problem with brute

force hybridization further research has focused on identifying the source of the resistance. Comparisons of blighted and unblighted trees along with American and Chinese chestnuts have led to several promising genes and pathways but there is further research that needs to be done (Barakat et al. 2009). Understanding blight resistance and crosses has allowed resurgence in the United States chestnut market including both Asian hybrids and modified American chestnuts.

### **Recovery and Economics**

The American chestnut market before the blight was economically critical, with timber in Pennsylvania, North Carolina, and West Virginia alone worth an estimated \$82.5 million in 1912 (Buttrick 1925, Anagnostakis 1987). Unfortunately, the American market was driven to near extinction due to the blight but has undergone a slow and steady recovery. A nationwide market survey in 2004 was conducted to gauge the progress of the recovery. The resulting data showed that the American chestnut industry is still in the beginning stages of a comeback (Gold et al. 2005). The survey showed that farming is a part-time occupation for over half the respondents and 83% of all respondents earn less than \$5000 a year. Also 64% had been producing for 10 years or less with the producers selling mostly to farmer's markets and on their own farms (Gold et al. 2005, 2006). The price per pound of chestnuts varied depending on where they were sold but mostly averaged between three to four dollars. Recent data are less clear about the status of the American chestnut market, but several things can be inferred such as the increase in price due to inflation, market growth unless demand significantly decreased, and more advanced advertising technology. Market growth has shown, however, that the blight is not the only factor to consider with increased production. Pest

insects for instance are particularly damaging because of the knowledge gap caused by the blight. The American chestnut market's growth has led to new problems emerging, but with the proper study the industry has the potential to continue growing.

### **The Lesser Chestnut Weevil**

#### **Biology**

There are two species of chestnut weevils in North America, the larger chestnut curculio, *Curculio caryatipes* Boheman, and the lesser or small chestnut weevil, *Curculio sayi* (Gyllenhal) (Brooks and Cotton 1929, Keeseey 2011). The lesser chestnut weevil, *C. sayi*, has been known by many synonyms including *Balaninius rectus*, *B. auriger mollis*, *B. strigosus*, *B. algonquinus*, *B. acuminatus*, *B. setosicornis*, *B. macilentus*, *B. perexillis*, and *Curculio auriger* (Johnson 1950). *Curculio* is a large genus with species across North America, Europe, Asia, and Africa (Hughes and Vogler 2004).

Adult *C. sayi* are chestnut pests that lay their eggs through burs into the nut tissue. The larvae emerge from the nuts after they drop (Brooks and Cotton 1929, Johnson 1950, Keeseey 2011). The larvae emerge by creating a hole of about 2 mm in diameter and then they burrow into the soil and create an earthen cell (Johnson 1950). Weevils remain in these cells for a two or three year obligate diapause at a depth usually less than 20 cm (Brooks and Cotton 1929, Keeseey and Barrett 2008). The larvae pupate for a period of two to three weeks before emerging as adults (Johnson 1950). Adults are mostly golden yellow with dark brown patches, but females later in the season can be dark brown to black in color. Additionally, adults are between 5-9 mm in length and 2.5 to 3.5 mm in width (Johnson 1950). Adult *C. sayi* are sexually dimorphic making them easily identified with males having rostrums from 2.5 to 3.5 mm and females having rostrums

from 5-9 mm (Brooks and Cotton 1929). Additionally, adult *C. sayi* appear to be adaptable and resilient as they managed to survive the destruction of American chestnuts by gravitating to pockets of uninfected trees, attacking young American chestnuts just able to produce nuts but not susceptible to blight, and finally perhaps by using chinquapin as an alternate host. Currently, *C. sayi* appears to have a range throughout Missouri, unlike *C. caryatrypes* that is only present in the southern region of Missouri (Keesey 2011).

### **Seasonal Emergence and Behavior**

Earlier studies of *Curculio sayi* have reported the weevil to have a single emergence period in early May (Brooks and Cotton 1929, Johnson 1950). Emergence of these adults coincides with the blooming of the catkins (spring florescence), a food source for the weevils. After this period the beetles disperse and are not found on the plants again until the middle of August during the maturation period of the burs (Johnson 1950). Research in mid-Missouri has confirmed this period of emergence but also reported another distinct period of adult weevil emergence (Keesey and Barrett 2008). The second (and smaller) emergence period occurs in late August coinciding closely with the return of the dispersed spring-emerging adults (Keesey 2011). For the destination of the spring emerged adults, it may be that the weevils migrate to an alternate host, but others suggest that it is more likely that adults return to the soil and ground debris (Brooks and Cotton 1929, Keesey 2011). Before dispersing, however, these *C. sayi* are fairly active in the canopy moving between catkins around every 30 minutes (Johnson 1950). A similar level of activity is present when the weevils return to mate and lay their eggs.

Several parasitoids have been observed attacking and have even been reared on *C. sayi* (Brooks and Cotton 1929). Female weevils drill a hole through the bur tissue to oviposit that is often taken advantage of by parasitoids sometimes immediately after the weevil eggs are deposited (Brooks and Cotton 1929). Male *C. sayi* aggregate around ovipositing females and have been observed charging at the parasitoids and chasing them off (Brooks and Cotton 1929). Chestnut weevils however are not usually larger or able to intimidate most of their natural enemies. Gray squirrels (*Sciurus sp.*) for instance feed on weevil larvae attacking as they emerge from the nuts, but also by digging weevils out of their earthen cells (Brooks and Cotton 1929). Weevils are attacked in a similar way by bobwhites (*Colinus virginianus virginianus*) that pick through nuts eating the emerging larvae (Brooks and Cotton 1929). Additionally, stomach content examination by the Bureau of Biological Survey found that more than 80 species of birds contained *Curculio* fragments (Brooks and Cotton 1929). These findings suggest that *C. sayi* have a broad range of natural enemies. Further support of this claim is the presence of dead adult weevils in spider webs of unknown species. Combating general predation, *C. sayi* have a similar behavioral response to other weevils, they feign death. When adult weevils perceive danger they feign death by pulling their limbs and mouthparts as close into their body and drop to the ground (if possible). Feigning death in this manner allows weevils to drop into the leaf litter and take full advantage of their cryptic coloration. Despite this defense mechanism, adult weevils around the time of oviposition have been observed and collected with broken rostrums (Johnson 1950). Without the rostrum, the sex of the damaged weevil cannot be easily determined, but a darker coloration and a relatively larger body size suggests female weevils (personal observation). This is consistent with

Johnson's explanation that the damage is caused by attacking birds, as female weevils would be vulnerable to attack when chewing a hole through bur tissue to lay their eggs (Johnson 1950).

### **Management Strategies**

Several control measures over time have been used to deal with the 'worms', a colloquial term for weevil larvae, in chestnuts. Two major angles have been approached to deal with the weevils based on attacking weevils at different stages. The first major approach is to attack weevils before they damage the nuts, and the second approach focuses on dealing with nuts after they are damaged in hope to salvage them. The limitation of the first strategy is that it is hard to produce a comprehensive solution that is specific to weevils, and the limitation of the second is that the damage is already done to the nuts. It has been reported that there is a difference in taste when chestnuts are damaged which makes the first approach more desirable (Johnson 1950). Avoiding pesticides or other invasive control methods that attack weevils before they damage the nut is advantageous economically, as nuts can be labeled organic (Gold et al. 2005). With these advantages in mind, another important factor to weigh is the secondary damage weevils cause as damaged nuts are more susceptible to fungal pathogens and weevils have shown synergistic effects with other pest species (Cooper and Rieske 2009, 2010). Combining pesticide application, clean practices, and prompt post-harvest treatments seems to be the most comprehensive solution to weevil damage (Hunt et al. 2009). Pesticides are appealing because they can target weaknesses in the weevil life cycle including the long diapause underground along with the specific timing of oviposition. Pesticides can thus be applied on the ground before adults emerge and on

the foliage before the weevils can oviposit. The Missouri Center for Agroforestry in its 2009 report “Growing Chinese Chestnuts in Missouri” suggests the use of carbaryl (Sevin®) in mid-August with three treatments at 10-day intervals but only on those orchards that need it (Hunt et al. 2009). Large established orchards are those most likely to require pesticides as the report points out that lesser chestnut weevil are still recovering and thus often take 10 to 15 years to find new chestnut plantings. In order to detect the presence of weevils and the severity of the infestation before harvest, limb-tapping or shaking to trigger the feigned death behavior of weevils can help estimate the extent of the problem. Additionally, when the infestation is not dire prompt post-harvest hot water treatment of nuts for 30 minutes at 100° F immediately cooling to 34° F can kill chestnut weevil eggs. However, hot water treatments are reported to alter the nut's nutritional content (Senter et al. 1994). But that tactic seems a far more palatable solution than larvae emerging from the nuts. Perhaps one of the most critical management tactics described is simply through the cultural practice of orchard sanitation (Payne et al. 1983). Prompt nut collection and the responsible disposal of damaged nuts can both directly lessen the amount of weevils in the orchard, prevent the spread of weevils to other chestnuts, and make weevils more vulnerable to their natural enemies. This effectiveness of these solutions is predicated on our knowledge of the *C. sayi* and that knowledge needs to be expanded. As *C. sayi* populations grow with the chestnut market alternative methods of management may be more efficient and effective.

## **Chemical Ecology in Plant-Insect Systems**

### **Host Plant Volatiles**



Phytophagous insects select their host through a variety of sensory cues, including visual cues like color or shape, gustatory (taste) cues after feeding on a potential host, and olfactory (smell) cues by interpreting the semiochemicals coming off the host (Visser 1986, 1988, Bruce et al. 2005). Olfactory cues in particular are critical as they travel long distances and insects can have an incredible amount of sensitivity to them. For example, sex pheromones for instance can be detected at remarkably low levels over great distances (Hansson 2002). Even a single molecule of a radiolabelled pheromone can be sufficient to elicit a nerve impulse in an insect's antennae (Kaissling 1986, Hansson 2002). Host plant volatiles have been shown to both synergize with pheromones (Light et al. 1993), as well as have their own high sensitivity and specificity (Hansson et al. 1999, Bruce et al. 2005). Evolutionary and ecological implications aside, host plant volatile organic compounds (VOCs) have had an interesting economic and practical impact. Disrupting host recognition or hijacking olfactory signals can be an effective method to monitor insect pests and control them (Visser 1986, Bruce et al. 2005). For example, several insects have been captured in monitoring traps using host plant VOCs; both combined with other attractants and applied alone in a trap (Visser 1986, Piñero and Prokopy 2003, Bruce et al. 2005). Several host plant VOCs of different groups including fatty acid derivatives, phenylpropanoids, and isoprenoids have been found to be electrophysiologically active among a large number of species in at least five different insect orders (Bruce et al. 2005). These volatiles seem to be conserved among different plants as the insect species tested have different host plants (Bruce et al. 2005). Additionally, many of the host plant fatty acid derivatives like (E)-2-hexenal, (E)-2-hexenol, and 2-heptanone have had similar responses in lesser chestnut weevils (Keeseey

et al. 2012). This evidence suggests that compound ratios are more likely to be the signal pest insects interpret for their host odor instead of a species-specific signal odor (Visser 1986, Bruce et al. 2005).

### **Electroantennography**

Most olfactory receptors on an insect are concentrated on the antennae (Hansson 2002, Bruce et al. 2005). Insect antennae serve to detect a wide variety of semiochemicals and relate them through neural impulses to the ganglia. Several electrophysiological techniques have been developed to decode the electrical signals generated by the antennae in hopes of determining the role of semiochemicals. The main tool employed by chemical ecologists is the electroantennogram (EAG). First developed during the study of the silk moth, *Bombyx mori*, the EAG revealed male antennal responses to the volatiles of female sex glands (Schneider 1957). Since its first introduction, however, the technique and equipment have become more sophisticated, but the basic components remain: a recording device, amplifier, and display (Roelofs 1984, Schneider and Seibt 1969, Struble and Arn 1984). An EAG works by capturing the electrical response of an antenna placed across two electrodes (one grounding and the other recording) that was stimulated with puffs of air containing selected volatiles at a known concentration. Once the signal is acquired it is passed through the amplifier allowing it to be displayed and measured. Several different antennal preparations have been developed including the excision of the entire head (Hibbard et al. 1997, Sullivan 2005), the insertion of pulled glass electrodes into the antennae (Altuzar et al. 2007), the use of a whole immobilized insect (Stelinski et al. 2003, Leskey et al. 2009), and most commonly the excision of a single antennae to be placed in saline or conductive gel

across a forked or two-plate probe (Jönsson and Anderson 1999, Keeseey and Barrett 2012, Keeseey et al. 2012). Preparations often depend on the antennae to be tested, for example weevil antennae have shown a characteristic concentration of olfactory receptors on their antennal clubs (Bland 1983, Alm and Hall 1986, Saïd et al. 2003) making the placement of the club in conductive gel problematic as it blocks these receptors. Two antennae have been used to compensate for this covered surface area (Keeseey 2011, Keeseey and Barrett 2012). Another common consideration when using EAG is antennal responses to the air movement caused by stimulus puffs. Aside from just olfactory receptors insects have mechanical receptors as well that respond to mechanical stimuli. When mentioned this air response is accounted for either by using air puff controls or adjustments to the baseline to negate the air puff responses (Stelinski et al. 2003, Wee et al. 2008, Szendrei et al. 2009).

### **Y-tube Olfactometry**

Behavioral bioassays rely on insects making a choice between stimuli based on their attractiveness or repellence. Olfactometers are the tools used when the stimuli are odors, such as plant VOCs. The different types of bioassays range in both the amount of stimuli provided and the way it is provided (Baker and Cardé 1984). Odor sources can use airflows, a consistent source and even provide concentration gradients. A Y-tube olfactometer combines the odor sources with a controlled bioassay. The Y-tube uses a constant rate of airflow over an odor source. Also, the Y-tube limits the stimuli to two odor sources allowing a direct comparison based on the deviation of choices away from a 50-50 choice split. Traditionally, Y-tube bioassays rely on using individual insects in sequential trials comparing the choices (Szendrei et al. 2009, Sun et al. 2010, Adesso et

al. 2011). Other behavioral bioassays rely on using multiple insects per trial and longer periods of time (van Tol et al. 2002). Further different types of odor sources can be used ranging from plant tissue to specific compounds of interest. A constant flow allows specific concentrations to be carried through the Y-tube to be compared to a control solution to evaluate attractiveness.

## CHAPTER II:

### EVALUATING CHESTNUT WEEVIL ELECTROANTENNOGRAM (EAG) RESPONSES TO DIFFERENT DOSES OF HOST PLANT VOLATILE ORGANIC COMPOUNDS (VOC)

#### Introduction

The lesser (or small) chestnut weevil, *Curculio sayi* (Gyllenhal), is a key economic pest of chestnut in the central and eastern regions of the United States (Brooks and Cotton, 1929). Host plants of this native weevil species are limited to only members of the genus *Castanea* (which include chestnut and chinquapin). It is reported to have a 2-3 year life cycle with populations having two annual periods of activity (Brooks and Cotton 1929, Johnson 1956, Keeseey and Barrett 2008). During the spring when the chestnut catkins are blooming adult weevils emerge from the ground and move into the trees and feed on the catkins. As the catkins enter senescence, the adult emergence from the soil decreases. Shortly thereafter the adult weevils leave the tree and, presumably, return to the ground debris where they enter a period of inactivity (Anagnostakis 2005).

When the chestnut burs begin to fully form and split (during late-summer/early-fall), the adult weevils return from their summer resting sites to the chestnut tree canopy. A second (but smaller) emergence of adults from the soil also occurs during this period (Keeseey and Barrett 2008). After mating the female weevil begins to lay eggs (usually in September) by chewing a hole through the nut and sometimes the bur. The developing larvae will feed on the nut contents for about 3 weeks, after which it will emerge from the nut and burrow into the soil to pupate (Brooks and Cotton 1929, Johnson 1956).

Electroantennography (EAG) is a technique often used because it provides a physiological baseline for weevil responses and a way to simplify complex behavioral

responses. Previous research has identified eight chestnut volatile organic compounds (VOCs) as being potentially attractive to *C. sayi*: 2-heptanol, (E)-2-hexen-1-ol, (E)-2-hexen-1-al, ethyl tiglate, ethyl butyrate, ethyl isobutyrate, ethyl-2-methyl butyrate, and 2-heptanone (Keeseey and Barrett 2012). The purpose of this study was to determine the antennal sensitivity of *C. sayi* to these key chestnut VOCs using electroantennography, at different doses, and to compare the EAG responses across weevil sex and season of adult activity.

## **Materials and Methods**

### **Field Site and Weevil Collection**

Adult *Curculio sayi* were collected from a private farm near the city of Glasgow within Saline County (39.19190° N, 92.93110° W), Missouri. Planted on the farm were several different nut trees, including several different chestnuts (*Castanea spp.*). The USDA soil type at the farm was Menfro silt loam. The site contains 14 chestnuts of different varieties spaced between 7 to 10 meters apart with overlapping canopies. The trees are 15-18 m tall and were estimated between 40 and 50 years of age and a grafted variety cross of Asian and American chestnut species (Ken Hunt, correspondence). The tree's catkins grow through April to June and nuts begin to drop in August continuing all the way through October.

*C. sayi* were collected using three types of traps: ground-based emergence traps, tree-mounted circle traps and silhouette traps (for detailed descriptions of trap specifics see Keeseey 2008). Additionally, a limb-tapping technique with canvas drop cloths to catch falling weevils dislodged from the canopy was employed. Once collected weevils were sexed using proboscis length and shape (a sexually dimorphic trait) and then

separated for transport back to the laboratory. In the laboratory the weevils were stored in plastic half liter cups containing 2-3 cm bedding of pine wood shavings. Two sponge cubes saturated with honey water were kept in each cup, which contained about 12 weevils (same sex). Cups were stored in a growth chamber set with a 14:10 (L:D) hour photoperiod and a temperature of 27° C. Collected weevils were tested only during the same period as their emergence, i.e. no spring weevils were tested or utilized during the fall testing period.

### **Antennal Preparation and EAG**

Antennal preparations and EAG procedures as described by Keeseey (2011) were followed. Such procedures that minimized baseline noise provided maximum sensitivity and lowered preparation time. For example, weevil antennae were excised by pulling them out at an angle perpendicular to the head. This procedure allows for the removal of internal neural connections with the antennae. Both antenna flagella were then placed across a forked probe (Syntech, Netherlands) and partially immersed in Spectra 360 electrode gel (Parker Laboratories, Inc., Fairfield, New Jersey) that was used as a connection medium. The probe was attached to a high-impedance electrometer and an indifferent grounding electrode (EAG Kombi Probe, type PRG-3; Syntech, Hilversum, Netherlands). An "x-y-z" coordinate micromanipulator (MP-15; Syntech, Hilversum, Netherlands) on a magnetic base was used to position the antennal preparation into a constant humidified and charcoal-purified air stream (0.5 liters/min). The air stream was carried through a 10 mm glass tube that flanged to 15 mm to contain the VOC preparation. An insertion point 13 cm down the glass tube away from the antennal preparation allowed odors to be delivered directly into the air stream using puff

cartridges. Puff cartridges consisted of a 15 cm borosilicate glass Pasteur pipette 6 mm in diameter containing a 1 x 1 cm disc of filter paper (Whatman No. 4) injected with 1  $\mu$ L of treatment solution. New puff cartridges were prepared for each antennal preparation and individual treatment (Figure 1). Three puffs of air, 1.0 second in duration (100 ml/min), were delivered at 30 second intervals with at least one minute between treatments and the replacement of a new puff cartridge. A stimulus flow controller (CS-55; Syntech, Hilversum, Netherlands) was used to manage both the constant airflow and air puffs (Figure 2).

### **Volatile Organic Compounds and Data Analysis**

The chestnut VOCs that were evaluated were: 2-heptanol, (E)-2-hexenol, (E)-2-hexenal, ethyl tiglate, ethyl butyrate, ethyl isobutyrate, ethyl-2-methyl butyrate, and 2-heptanone. (E)-2-hexenol was identified as only coming from chestnut catkins, while (E)-2-hexenal, 2-heptanone and 2-heptanol were found from the catkin and burr tissue. The esters were all identified as coming from the nut tissue alone, except for ethyl isobutyrate, which was produced by the bur tissue as well. Treatment solutions were prepared at four different dilutions for each compound: 1:10, 1:100, 1:1,000, and 1:10,000 with the solvent being laboratory grade mineral oil (Sigma Aldrich, St. Louis, MO). Weevils collected in the spring were split into two groups: the first containing the highest two doses for all VOCs and the second the lower two doses for all VOCs. However, the fall weevils were split into four groups halving the groups of spring with each group containing all volatiles of a single dose. Groups were tested by randomizing the order of the eight volatiles using a random number table and then testing the treatments in series of a single dose with two dose groups beginning with the lower of the doses. Control



solutions were puffed at the beginning and end of each recording with an additional control between the doses of spring groups. Antennal responses were recorded using Syntech software (GC-EAD 2010) after they were passed through a high-impedance amplifier in a two-channel acquisition system optimized for EAG signals (IDAC-2; Syntech, Hilversum, Netherlands) (Figure 2). Noise was controlled using a 150 kg steel plate base (3" x 12" x 18") placed on a 2 cm thick rubber pad as the attachment point for the antennal preparation. The sex and season of activity for adult weevils was recorded along with the antennal responses. Antennal response amplitude was measured as absolute value difference from the baseline.

The data were analyzed with a 3-way analysis of variance (ANOVA) (PROC MIXED; SAS v. 9.3 SAS Institute Inc., Cary, NC) in two separate groups for spring-active and fall-active weevils. The explanatory variables examined were compound, dose and sex of the weevil. Data from spring-active weevils were treated as a split-split plot with the main plot consisting of sex of the weevil, the subplot of sex and sex by dose and the sub-subplot of compound with all possible interactions with the main and subplots. Data from fall-active weevils were treated as a split plot with the main plot consisting of sex, dose and sex by dose. The subplot included compound along with all possible interactions with the main plot. Mean differences for both spring and fall-active weevils were determined using least-squares means (PROC MIXED; LS MEANS: SAS v. 9.3).

## **Results**

The analysis of variance (ANOVA) performed on spring-active weevils showed significant individual effects of all three individual variables considered (weevil sex, compound and dose) (Table 1). Additionally, there were significant interactions present

between compound and sex, compound and dose, and a three-way interaction between compound, sex and dose. Significance was evaluated at a level of  $P < 0.05$  for the effects tested.

The ANOVA performed on fall-active weevils showed significant individual effects of two individual variables, compound and dose (Figure 3). Additionally there were significant interactions present between compound and sex, compound and dose, and a three-way interaction between compound, sex and dose. Significance was evaluated at a level of  $P < 0.05$  for the effects tested.

### **Spring Weevil EAG Responses**

Electroantennogram (EAG) responses from spring active adult *C. sayi* were averaged and calculated as relative percentages to their respective controls. For females exposed to the (E)-2-hexenol treatment, the mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) were 4.66 mV (15.82%), 3.61 mV (10.02%), 5.89 mV (33.68%), and 5.01 mV (32.88%), respectively (Figure 3). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 6.19 mV (42.8%), 4.35 mV (16.92%), 6.48 mV (50.01%), and 5.15 mV (37.46%), respectively (Figure 3).

The mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) for female weevils exposed to the 2-heptanol treatment were 3.97 mV (-1.42%), 3.38 mV (2.91%), 5.85 mV (33%) and 5.19 mV (37.59%), respectively (Figure 4). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest

dose were 4.42 mV (2.02%), 3.88 mV (4.37%), 4.94 mV (14.46%), and 5.15 mV (37.55%), respectively (Figure 4).

For female weevils exposed to the (E)-2-hexenal treatment, the mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) were 3.84 mV (-4.63%), 3.52 mV (7.2%), 5.19 mV (17.8%), and 4.6 mV (21.97%), respectively (Figure 5). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 3.85 mV (-11.21%), 4.31 mV (15.79%), 7.13 mV (65.12%), and 5.46 mV (45.65%), respectively (Figure 5).

The mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) for female weevils exposed to the 2-heptanone treatment were 3.97 mV (-1.41%), 3.32 mV (7.2%), 5.24 mV (17.8%), and 5.11 mV (35.66 %), respectively (Figure 6). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 4.34 mV (0.08%), 4.01 mV (7.68%), 5.54 mV (28.26%), and 6.09 mV (62.42%), respectively (Figure 6).

For female weevils exposed to the ethyl butyrate treatment, the mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) were 3.82 mV (-5.05%), 3.43 mV (4.54%), 4.88 mV (10.81%), and 4.89 mV (29.61%), respectively (Figure 7). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 4.13 mV (-4.64%), 3.75 mV (0.75%), 4.86 mV (12.69%), and 5.49 mV (46.46%), respectively (Figure 7).

The mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) for female weevils exposed to the ethyl-2-methyl butyrate treatment were 4.24 mV (5.37%), 3.53 mV (7.57%), 5.6 mV (27.12%), and 4.96 mV (31.49%), respectively (Figure 8). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 4.12 mV (-4.99%), 3.86 mV (3.83%), 6.44 mV (49.26%), 5.79 mV (54.47%), respectively (Figure 8).

For female weevils exposed to the ethyl tiglate treatment, the mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) were 3.59 mV (-10.72%), 3.23 mV (-1.81%), 4.62 mV (4.99%), and 4.91 mV (30.23%), respectively (Figure 9). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 4.02 mV (-7.35%), 3.79 mV (1.9%), 5.11 mV (18.47%), and 6.05 mV (61.54%), respectively (Figure 9).

The mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) for female weevils exposed to the ethyl isobutyrate treatment were 4.1 mV (1.78%), 3.38 mV (3.02%), 4.85 mV (9.98%), and 4.51 mV (19.7%), respectively (Figure 10). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 4.3 mV (-0.77%), 3.8 mV (2.01%), 5.18 mV (20%), 4.49 mV (19.68%), respectively (Figure 10).

The mean antennal EAGs from female weevils responded significantly at a level of  $P < 0.05$  to all compounds at the highest dose while only (E)-2-hexenol, 2-heptanol,

(E)-2-hexenal, 2-heptanone, and ethyl-2-methyl butyrate received significant responses at the 1:10 dose. The 1:1000 and 1:10000 doses among all eight compounds did not receive statistically significant responses from female *C. sayi*. The controls shared among the compounds but within doses had average amplitudes of 4.02, 3.29, 4.41, and 3.77 mV for the 1:10, 1:100, 1:1000, and 1:10000 dilutions, respectively (Figures 11-14).

For the males, the mean antennal EAGs were significant ( $P < 0.05$ ) at the highest dose to all compounds like female weevils; however, at the 1:100 dose they instead responded to (E)-2-hexenol, (E)-2-hexenal, 2-heptanone, ethyl-2-methyl butyrate, ethyl tiglate and ethyl isobutyrate. (E)-2-hexenol was the only compound that received significant responses at the lower two doses responding overall responding at all 4 doses. The controls shared among the compounds but within doses had average amplitudes of 4.34, 3.72, 4.32, and 3.75 mV for the 1:10, 1:100, 1:1000, and 1:10000 dilutions respectively (Figures 11-14).

### **Fall Weevil EAG Responses**

Electroantennogram (EAG) responses from fall active adult *C. sayi* were averaged and calculated as relative percentages to their respective controls. For females exposed to the (E)-2-hexenol treatment, the mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) were 6.07 mV (-1.99%), 4.35 mV (60.15%), 3.74 mV (9.19%), and 5 mV (53.64%), respectively (Figure 3). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 6.7 mV (9.36%), 4.69 mV (22.69%), 4.93 mV (31.05%), and 5.52 mV (63.73%), respectively (Figure 3).

The mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) for female weevils exposed to the 2-heptanol treatment were 5.8 mV (-6.27%), 3.12 mV (14.98%), 5.04 mV (47.2%), and 4.94 mV (51.74%), respectively (Figure 4). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 5.36 mV (-12.56%), 4.06 mV (6.22%), 4.87 mV (29.45%), and 5.09 mV (51.1%), respectively (Figure 4).

For female weevils exposed to the (E)-2-hexenal treatment, the mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) were 6.2 mV (0.14%), 3.43 mV (26.42%), 3.64 mV (6.26%), 5.98 mV (83.77%), respectively (Figure 5). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 5.92 mV (-3.41%), 5.16 mV (34.89%), 5.31 mV (41.26%), and 6.07 mV (79.95%), respectively (Figure 5).

The mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) for female weevils exposed to the 2-heptanone treatment were 5.61 mV (-9.43%), 3.43 mV (26.21%), 3.54 mV (3.49%), and 8.4 mV (158.09%), respectively (Figure 6). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 5.82 mV (-5%), 3.98 mV (4.09%), 5.01 mV (33.13%), and 6.17 mV (82.97%), respectively (Figure 6).

For female weevils exposed to the ethyl butyrate treatment, the mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in

parentheses) were 5.82 mV (-5.99%), 2.65 mV (-2.65%), 4.49 mV (31.24%), and 5.01 mV (53.98%), respectively (Figure 7). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 5.4 mV (-11.93%), 4 mV (4.26%), 4.41 mV (17.32%), and 6.35 mV (88.47%), respectively (Figure 7).

The mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) for female weevils exposed to the ethyl-2-methyl butyrate treatment were 6.31 mV (1.89%), 3.11 mV (14.7%), 4.14 mV (21.02%), and 7.66 mV (135.47%), respectively (Figure 8). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 6.17 mV (0.74%), 4.16 mV (8.74%), 5.27 mV (40.17%), 4.54 mV (34.59%), respectively (Figure 8).

For female weevils exposed to the ethyl tiglate treatment, the mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) were 5.96 mV (-3.72%), 2.73 mV (0.48%), 3.95 mV (15.4%), and 7.74 mV (137.73%), respectively (Figure 9). For male weevils exposed to this same VOC treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 5.94 mV (-3.13%), 3.9 mV (1.97%), 4.62 mV (22.81%), and 7.44 mV (120.76%), respectively (Figure 9).

The mean EAG responses at the 0.0001, 0.001, 0.01 and 0.1 doses (with the relative percentages in parentheses) for female weevils exposed to the ethyl isobutyrate treatment were 5.76 mV (-6.91%), 3.06 mV (12.77%), 3.82 mV (11.48%), and 5.74 mV (76.44%), respectively (Figure 10). For male weevils exposed to this same VOC

treatment, the mean EAG responses (and relative percentages) from the lowest to highest dose were 5.58 mV (-8.94%), 4.13 mV (8.01%), 4.56 mV (21.18%), 4.15 mV (23.06%), respectively (Figure 10).

All compounds received significant responses ( $P < 0.05$ ) for mean antennal EAGs for females at the highest dose, but from no other treatments besides (E)-2-hexenol at the 1:1000 dose. The controls shared among the compounds but within doses had average amplitudes of 6.19, 2.72, 3.42, and 3.25 mV for the 1:10, 1:100, 1:1000, and 1:10000 dilutions respectively (Figures 11-14).

For the males, (E)-2-hexenol, 2-heptanol, (E)-2-hexenal, 2-heptanone, ethyl butyrate, and ethyl tiglate all received significant EAG responses ( $P < 0.05$ ) at the highest dose but only (E)-2-hexenal at 1:1000 and ethyl-2-methyl butyrate at 1:100 received significant responses ( $P < 0.05$ ) as well. The controls shared among the compounds but within doses had average amplitudes of 6.13, 3.83, 3.76, and 3.37 mV for the 1:10, 1:100, 1:1000, and 1:10000 dilutions respectively (Figures 11-14).

## Discussion

Several VOCs showed strong antennal responses at the higher doses as expected, and for many other compounds a detection threshold can be inferred. Among all the VOCs at each combination of sex and season, only (E)-2-hexenol received significant responses at all doses and only from spring emerging males. Besides the 1:10 dose, the responsiveness for almost all compounds was less widespread especially in the fall emerging weevils. Fall weevils only responded to three compounds at the lower three doses with females responding to (E)-2-hexenol at 1:1000 and males responding to (E)-2-hexenal at 1:1000 and ethyl-2-methyl butyrate at 1:100. The difference in sensitivity



between the two emergence periods is perhaps most obvious between males. For instance the response to ethyl isobutyrate did not differ significantly from the control in the fall and there was less significant differences at lower doses between all compounds and their controls except 2-heptanol and ethyl butyrate. Females also seem to be less responsive in the fall but the effect was less widespread, with the main difference being the greater antennal response to (E)-2-hexenal at the 1:100 dose. Females between seasons did continue to respond to the highest doses for all compounds so rather than responsiveness in general it could be a matter of antennal sensitivity. The EAG responses for the control treatments between the two emergence periods were less erratic in the spring with mean control responses for each sex at each dose being around 4 mV, while in the fall, most of the control responses were a little smaller except for two large controls at the lowest dose for both sexes at over 6 mV each. The sensitivity of the antennae was accounted for in the recordings but may be an indication in and of itself of physiological changes between seasons that could explain the different antennal responses. The magnitude of responses, however, are less clear when it comes to differences between seasons with fairly uniform responses at the lower doses but several extremely large responses at the highest doses from fall weevils. Fall weevils responded at over 100% to 3 different compounds with females at 158.09% and 135.47% for 2-heptanone and ethyl-2-methyl butyrate while ethyl tiglate got 137.73% from females and 120.76% from males. The largest response regardless of compound, sex, or dose in the spring season was only 65.12% to (E)-2-hexenal at 1:100 from males. The antennae in fall while detecting less doses of each compound seem to produce larger responses when the compounds were detected. Unfortunately due to the change in protocol between

seasons more direct comparisons are not viable. After the completion of the spring season of data collection the protocol was changed to shorten recordings in order to prevent antennal decay within recordings. Out of the four doses, the lower two tended not receive responses while the highest dose received responses from nearly every grouping of weevil regardless of compound. The general similarity of doses between compounds however is not representative of all compounds. (E)-2-hexenol seems to break the pattern for each sex and season combination except fall males; fall females responded just as strongly to the 1:1000 dose at the 1:10, the spring males responded to all doses except 1:1000 strongly, and spring females responded to the 1:10 and 1:100 doses equally. (E)-2-hexenal, ethyl-2-methyl butyrate, and ethyl isobutyrate each at one sex and season combination showed difference from the increase in response expected as dose increased. Comparisons between compounds at the lower two doses did not reveal much as most of the compounds were not significantly different than the control but at the 1:1000 dilution several compounds were close to significant and not different than the responses that were. The six-carbon compounds, (E)-2-hexenol and (E)-2-hexenal, were most often significant at the lower doses had a similar effect at the 1:100 dose. Spring-active males showed larger responses to (E)-2-hexenal than any other compound while (E)-2-hexenol and ethyl-2-methyl butyrate were larger than the rest as well but not (E)-2-hexenal. Spring females and fall males at the 1:100 dose showed smaller differences but fall females showed no difference between compounds. At the highest dose spring-active weevils showed less differences between compounds while fall weevils had several compounds that responded strongly. Among the fall females 2-heptanone, ethyl-2-methyl butyrate, and ethyl tiglate received the similar large responses while the other

compounds received smaller responses. Fall males received similar large responses from most of the compounds but ethyl-2-methyl butyrate and ethyl isobutyrate received smaller responses. Ethyl tiglate specifically received large responses from fall male weevils at the 1:10 dose. *C. sayi* responded to esters at the highest dose strongly but not as strongly to lower doses. The response of weevils was more even at the lower doses to the rest of the compounds and specifically (E)-2-hexenol and (E)-2-hexenal. (E)-2-hexenol, (E)-2-hexenal, 2-heptanone, and ethyl butyrate were selected at the 1:10 dose for further study in mixtures. Although some other compounds had larger magnitude responses, those included are a mixture of different chemical structures, responsive at the selected dose, and present in the literature as potential attractants (Dickens 1989, 1989, Leskey et al. 2001, Toshova et al. 2010, Guarino et al. 2011).

The vine weevil, *Otiorhynchus sulcatus* Fabricius, showed similar physiological responsiveness to (E)-2-hexenol and (E)-2-hexenal in a logarithmic dose-response with significant responses through the  $10^{-4}$  dilution (van Tol and Visser 2002). Additionally the largest response among vine weevils to the individual volatiles was at the largest dose which was the  $10^{-1}$  dilution. Toshova et al. in 2010 evaluated (E)-2-hexenol as well for the grey corn weevil, *Tanymecus (Episomecus) dilacollis* Gyllenhal, and found fairly similar results in both the range of responsiveness and strength of response. *C. sayi*, like other weevils, appears to be more responsive at lower concentrations to fatty acid derivatives ((E)-2-hexenol, 2-heptanol, etc.) than esters or other compounds considering both the EAG dose-response of the selected VOCs and the preliminary dose-response performed by Keeseey (2011). The sensitivity of weevils to these VOCs suggests that if they are important for host location then concentration may be a factor in the strength or

presence of an attractive behavioral response. Although increased antennal responses don't necessarily lead to increased behavioral responses other factors in the antennal recordings may provide clues to potential behavioral responses. In the preliminary dose-response EAG screening of VOCs on *C. sayi*, Keeseey (2011) reports a change in the sign of the antennal responses with large negative responses from (E)-2-hexenal at the largest dose when compared to positive amplitude responses at lower doses. Another factor that may provide more insight into potential behavioral responses is the presence of bimodal antennal responses. Neither of these factors appeared to follow a pattern in our data and were therefore controlled with using absolute values and the selection of the largest response peak.

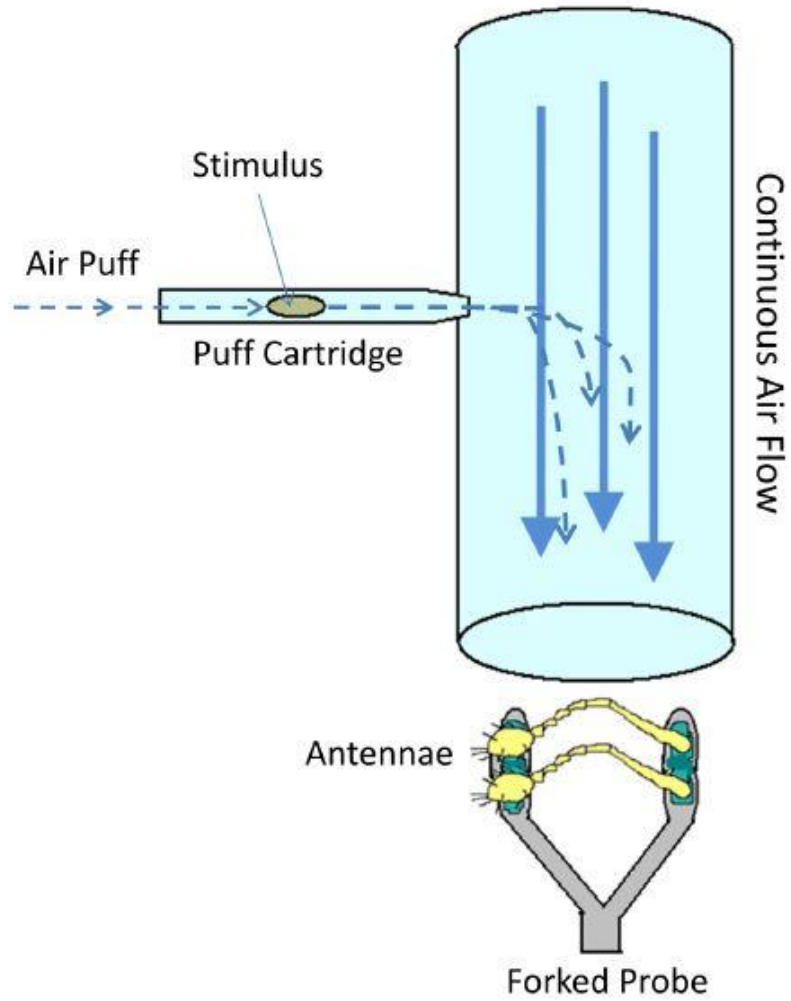
### Spring EAG Results

Effect	Num DF	Den DF	F value	Pr > F
<b>Sex</b>	1	198	4.30	0.0394
<b>Compound</b>	7	1383	14.32	<.0001
<b>Dose</b>	3	198	10.97	<.0001
Compound*Sex	7	1383	6.13	<.0001
Sex*Dose	3	198	1.22	0.3050
Compound*Dose	21	1383	7.19	<.0001
Compound*Dose*Sex	21	1383	3.37	<.0001

### Fall EAG Results

Effect	Num DF	Den DF	F value	Pr > F
<b>Sex</b>	1	70	0.08	0.7823
<b>Compound</b>	7	490	3.80	0.0005
<b>Dose</b>	3	70	7.45	0.0002
Compound*Sex	7	490	2.65	0.0108
Sex*Dose	3	70	0.23	0.8774
Compound*Dose	21	490	6.12	<.0001
Compound*Dose*Sex	21	490	3.42	<.0001

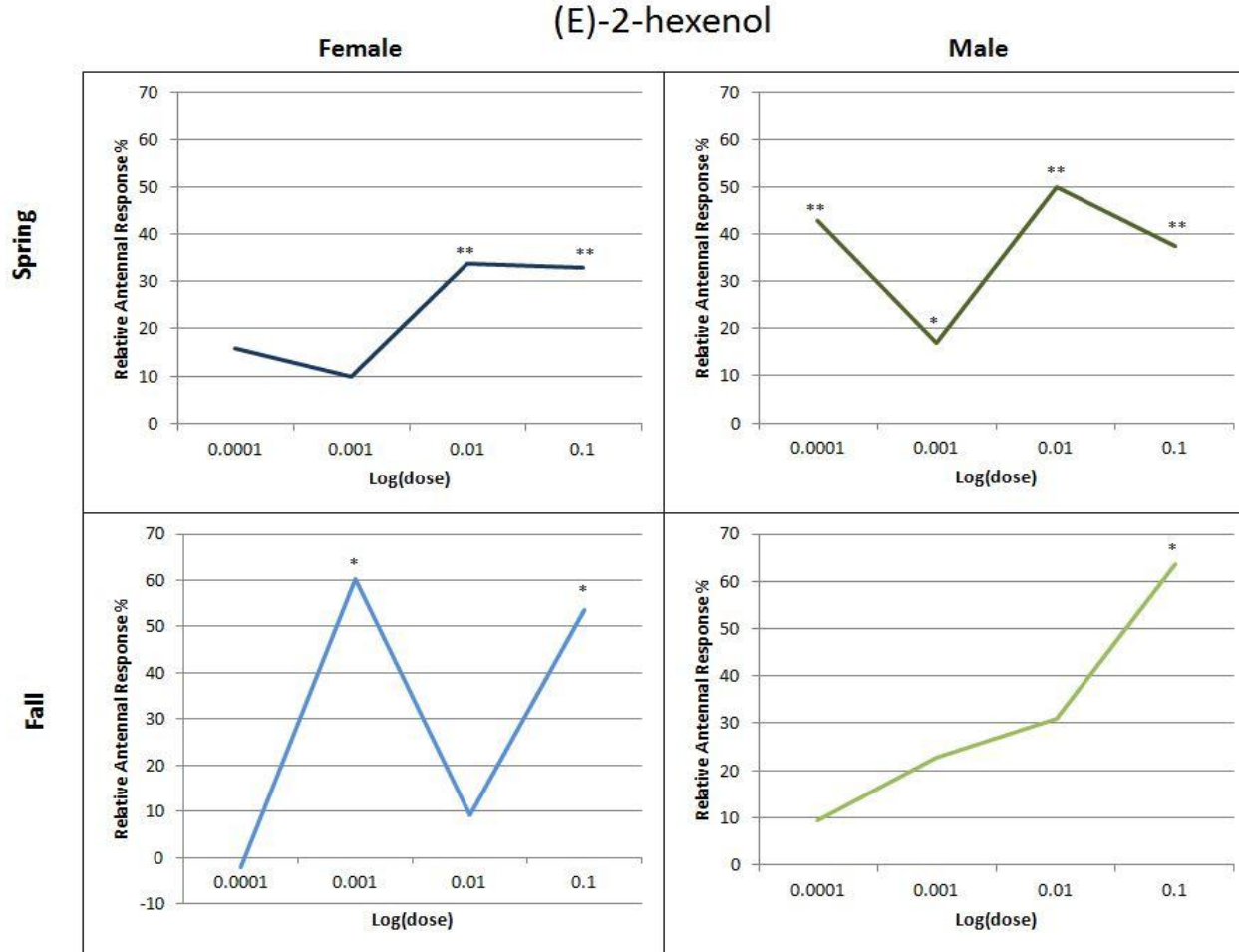
**Table 1.** Results of ANOVA performed on beetle sex, treatment compound, and dose of compound per spring-active and fall-active weevil data from EAG.



**Figure 1.** Diagram of the weevil antennal preparation (Keesey 2011).

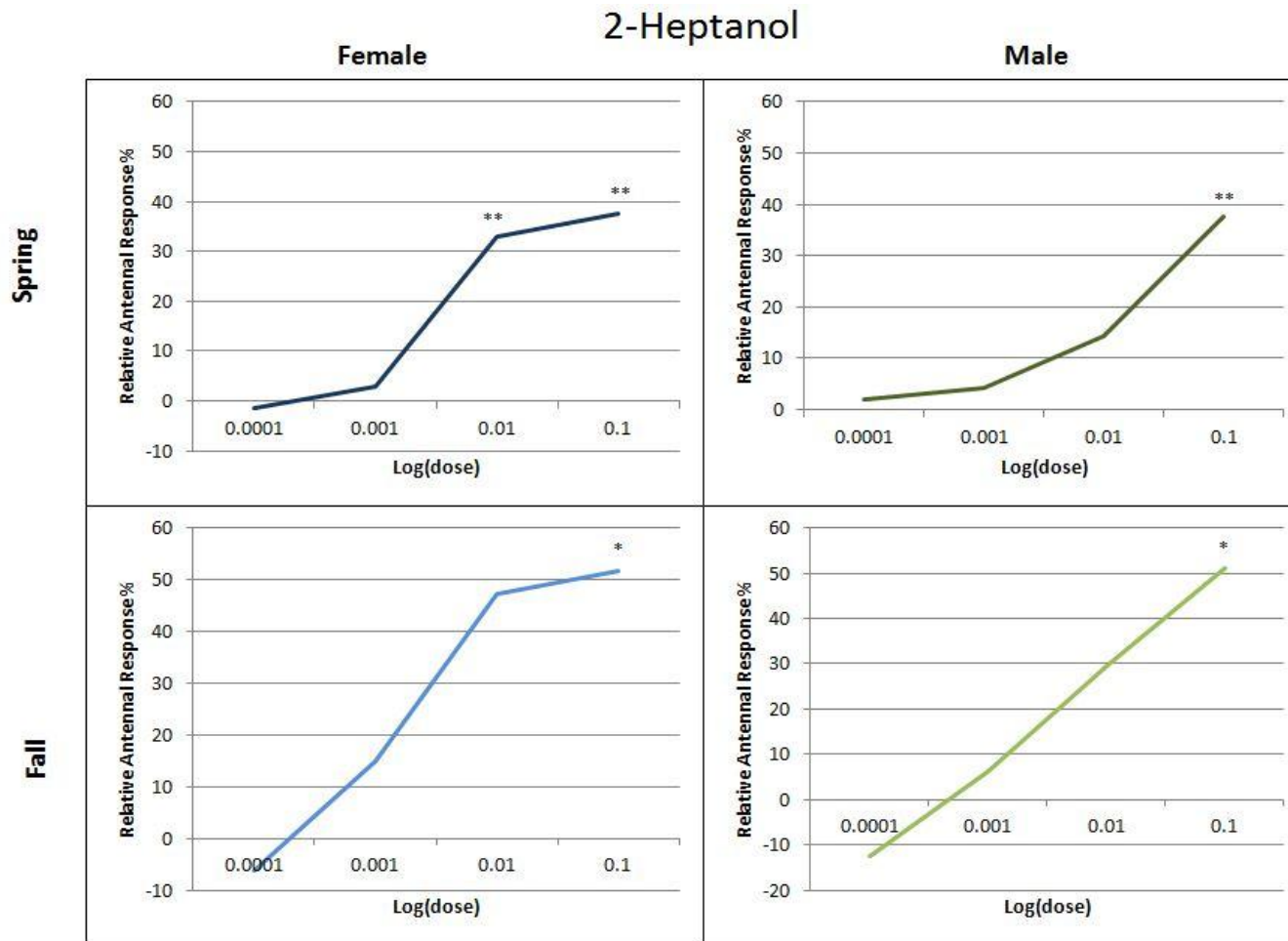


**Figure 2.** Electroantennogram (EAG) equipment. Top left: stimulus flow controller. Top right: 3 dimensional micromanipulators. Bottom left: continuous airflow tube connected to GC-EAD equipment. Bottom right: IDAC-2 high-impedance amplifier and two-channel controller (images from Syntech, Hilversum, Netherlands).

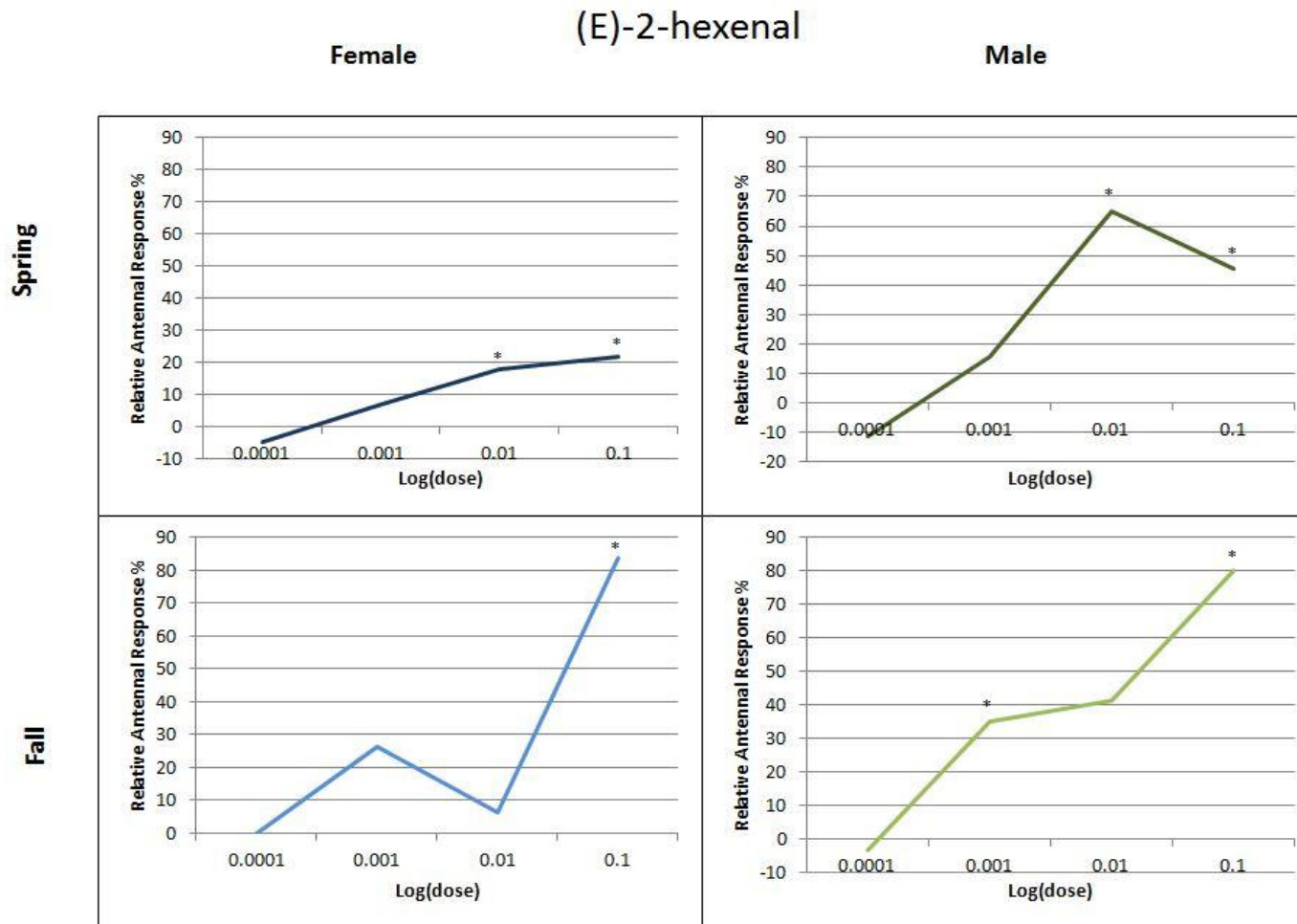


**Figure 3.** The mean antennal responses of each group of *C. sayi* to (E)-2-hexenol at all four doses. The presence of an asterisk(s) per dose, sex and period of weevil seasonal activity denote which antennal responses are significantly different from their respective control responses (Fisher's protected LSD; \* $P < 0.05$ , \*\* $P < 0.0001$ ).

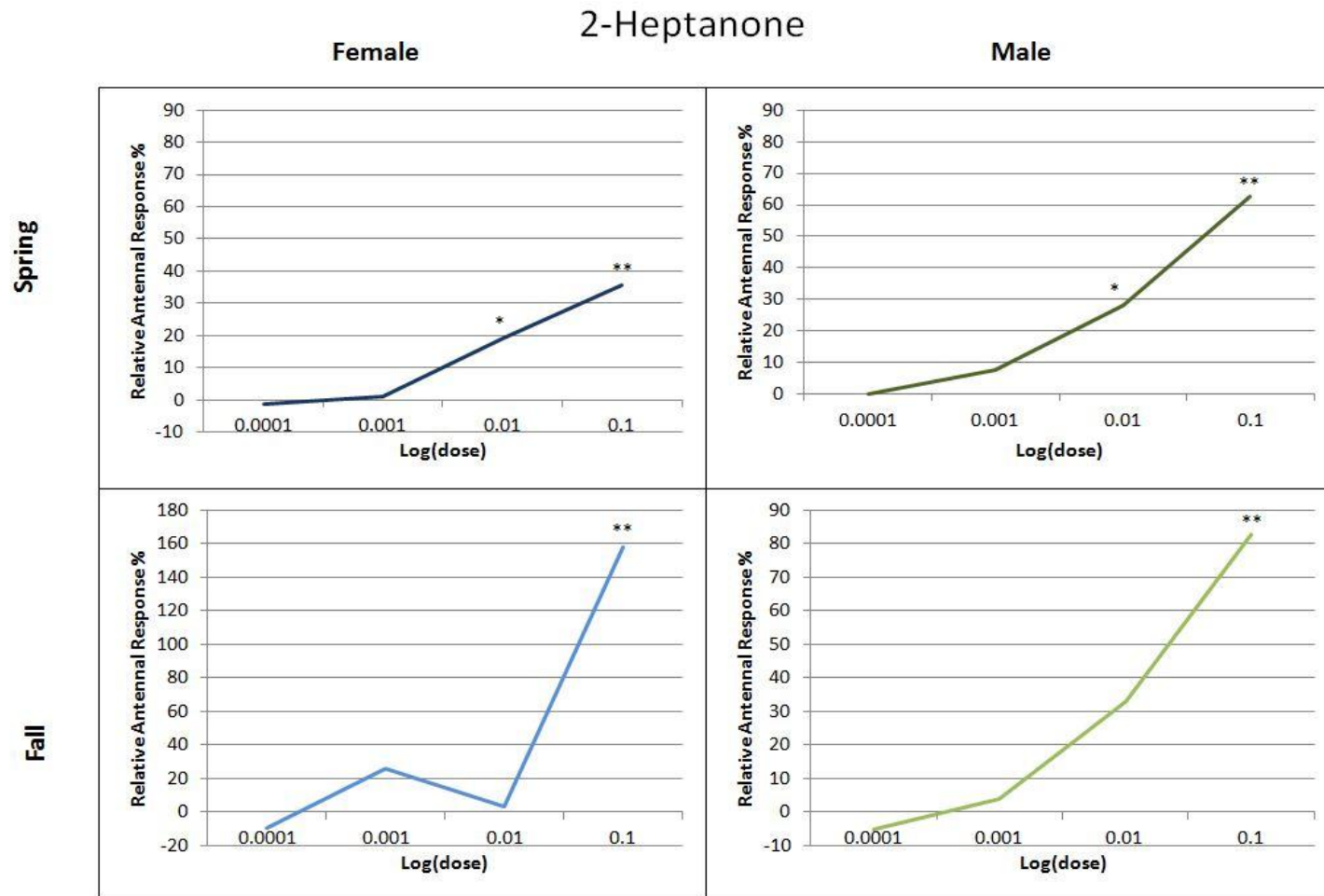




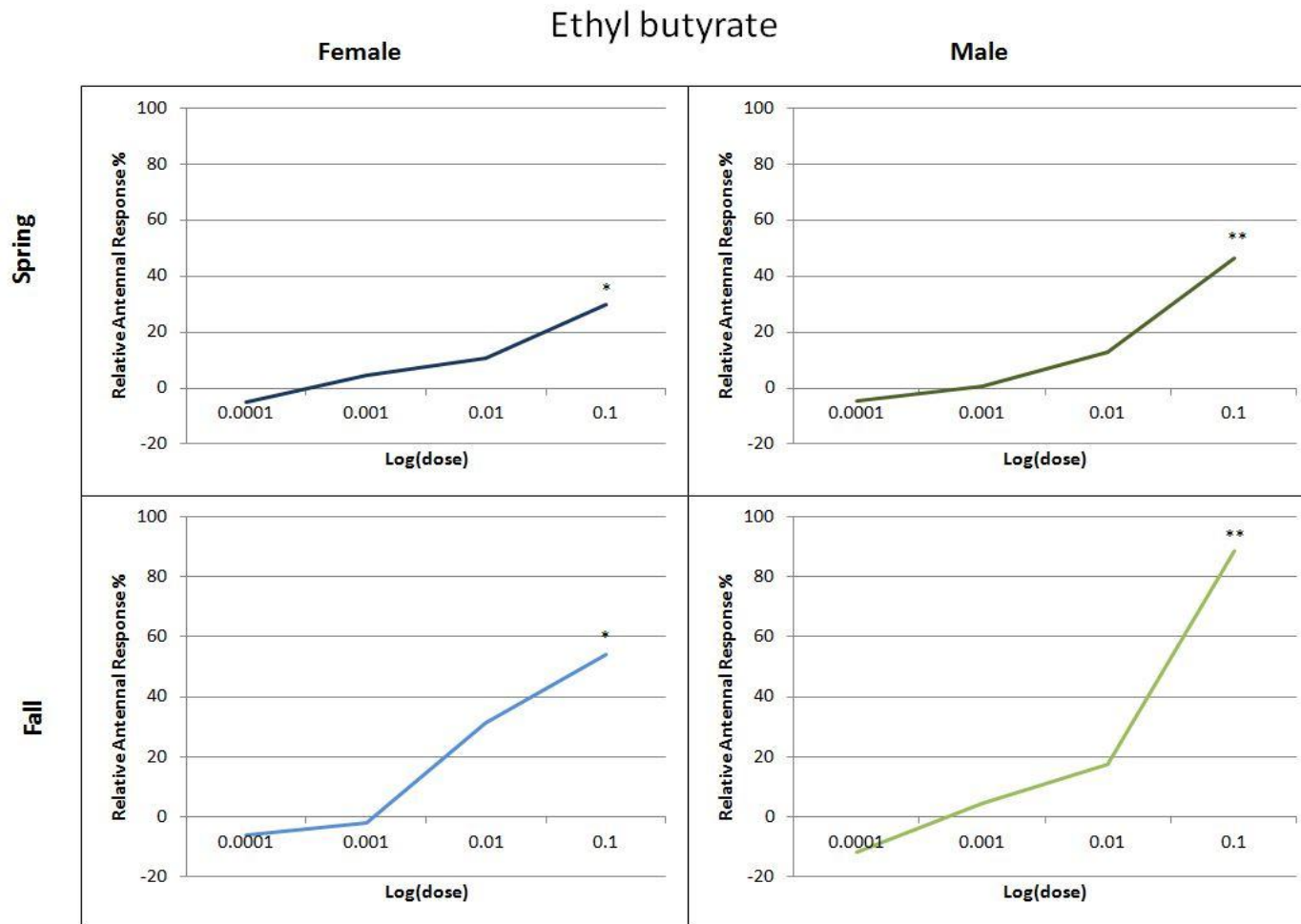
**Figure 4.** The mean antennal responses of each group of *C. sayi* to 2-heptanol at all four doses. The presence of an asterisk(s) per dose, sex and period of weevil seasonal activity denote which antennal responses are significantly different from their respective control responses (Fisher's protected LSD; \*P < 0.05, \*\*P < 0.0001).



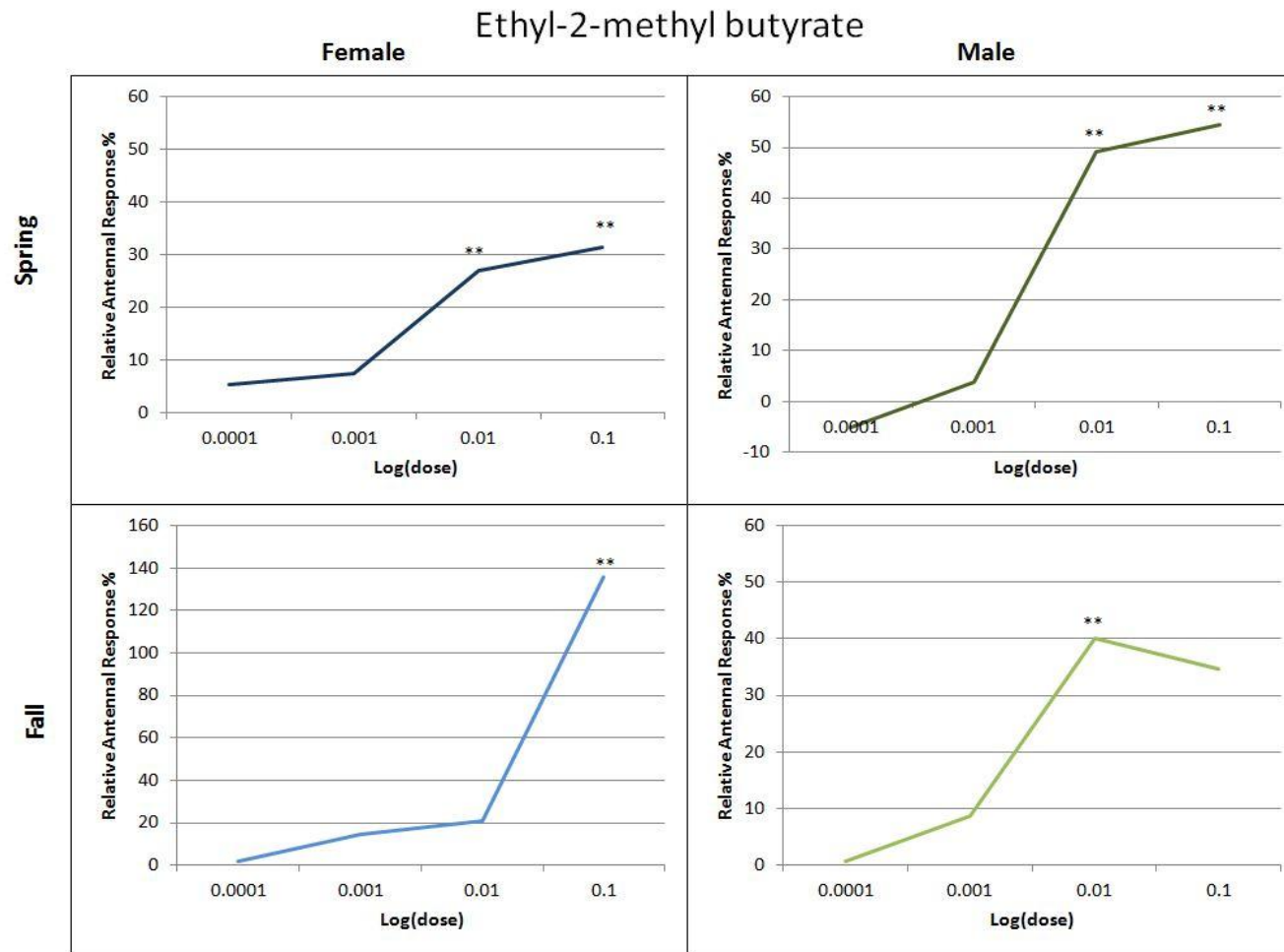
**Figure 5.** The mean antennal responses of each group of *C. sayi* to (E)-2-hexenal at all four doses. The presence of an asterisk(s) per dose, sex and period of weevil seasonal activity denote which antennal responses are significantly different from their respective control responses (Fisher's protected LSD; \*P < 0.05, \*\*P < 0.0001).



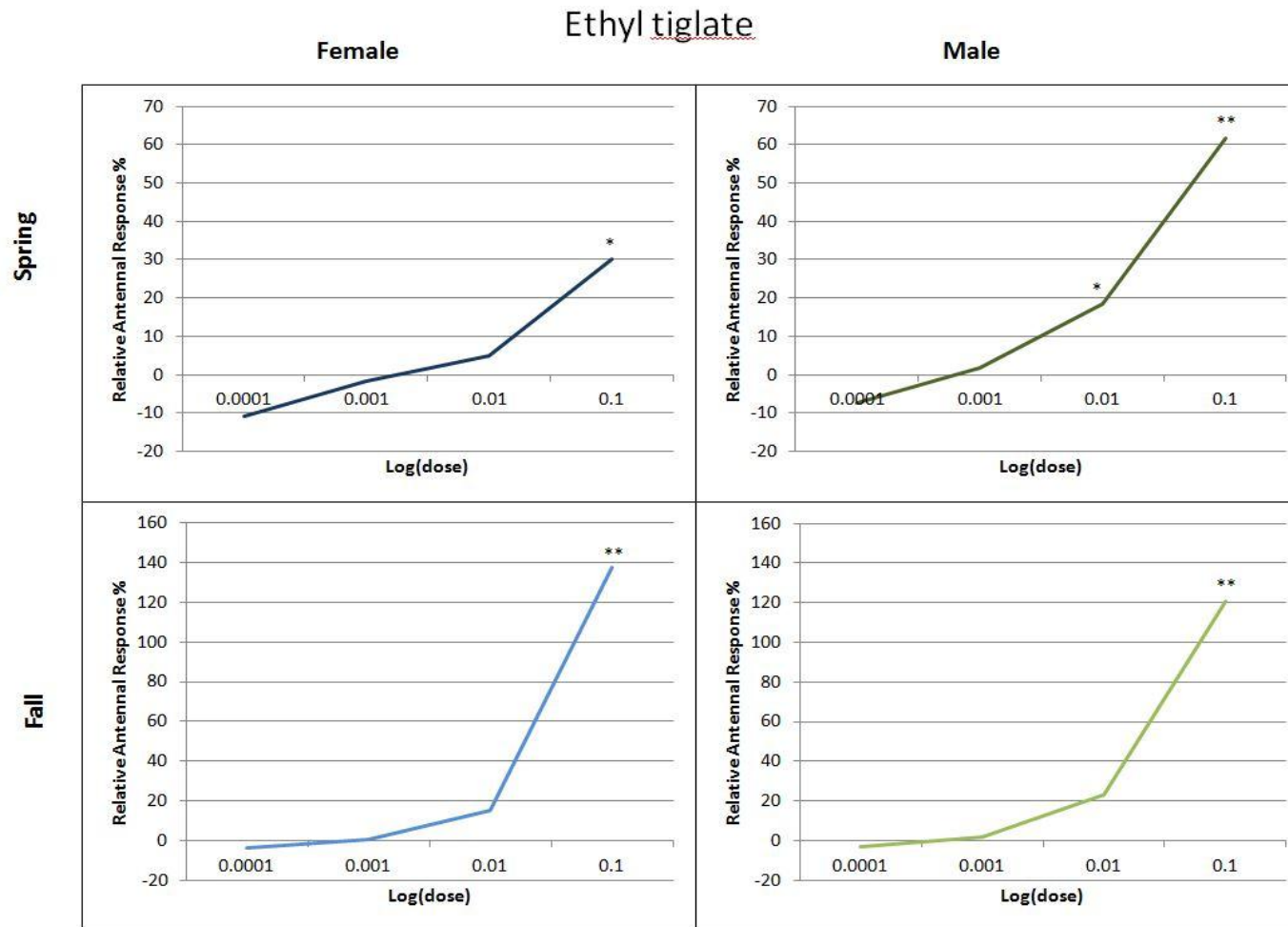
**Figure 6.** The mean antennal responses of each group of *C. sayi* to 2-heptanone at all four doses. The presence of an asterisk(s) per dose, sex and period of weevil seasonal activity denote which antennal responses are significantly different from their respective control responses (Fisher's protected LSD; \* $P < 0.05$ , \*\* $P < 0.0001$ ).



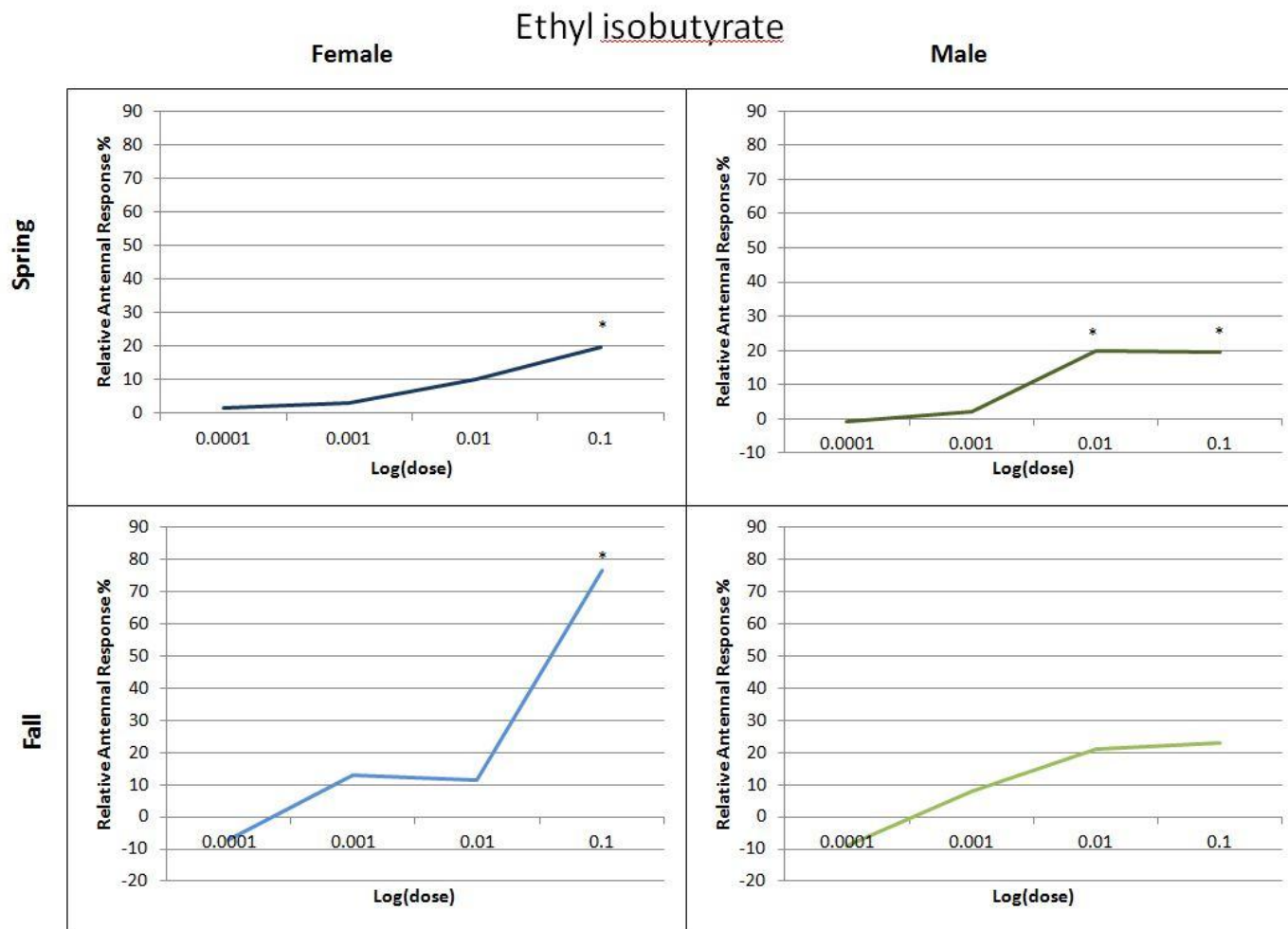
**Figure 7.** The mean antennal responses of each group of *C. sayi* to ethyl butyrate at all four doses. The presence of an asterisk(s) per dose, sex and period of weevil seasonal activity denote which antennal responses are significantly different from their respective control responses (Fisher's protected LSD; \* $P < 0.05$ , \*\* $P < 0.0001$ ).



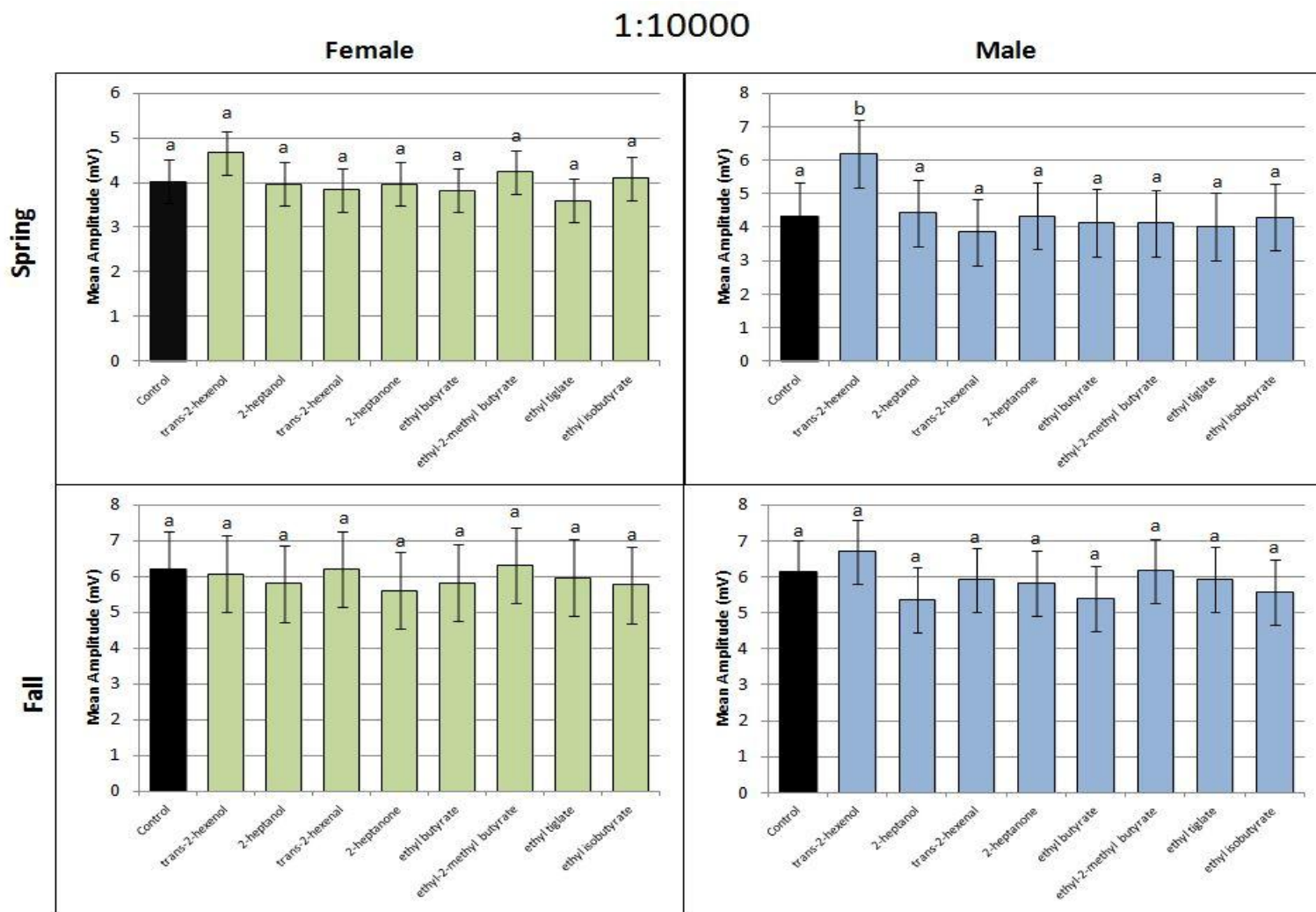
**Figure 8.** The mean antennal responses of each group of *C. sayi* to ethyl-2-methyl butyrate at all four doses. The presence of an asterisk(s) per dose, sex and period of weevil seasonal activity denote which antennal responses are significantly different from their respective control responses (Fisher's protected LSD; \* $P < 0.05$ , \*\* $P < 0.0001$ ).



**Figure 9.** The mean antennal responses of each group of *C. sayi* to ethyl tiglate at all four doses. The presence of an asterisk(s) per dose, sex and period of weevil seasonal activity denote which antennal responses are significantly different from their respective control responses (Fisher's protected LSD; \*P < 0.05, \*\*P < 0.0001).

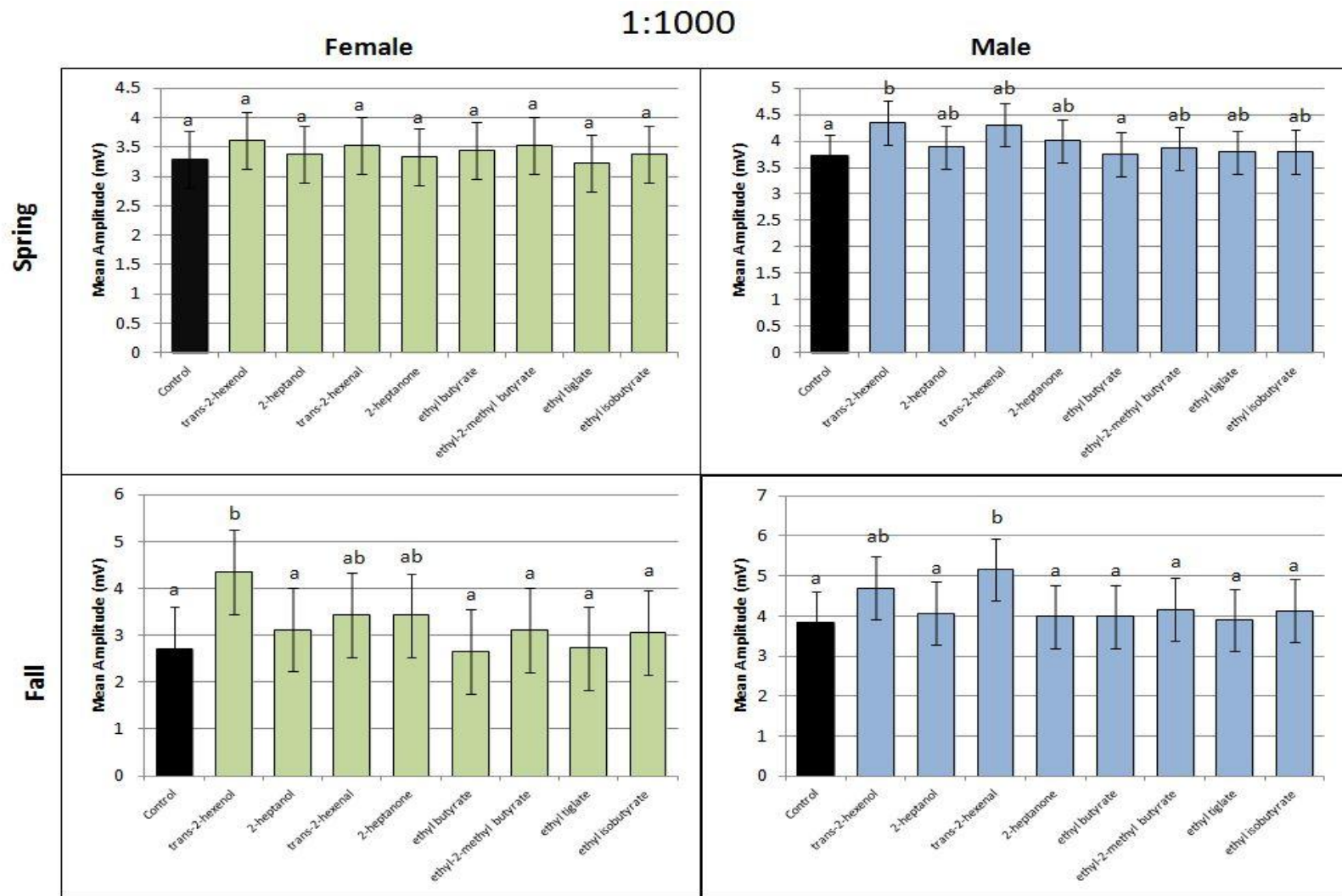


**Figure 10.** The mean antennal responses of each group of *C. sayi* to ethyl isobutyrate at all four doses. The presence of an asterisk(s) per dose, sex and period of weevil seasonal activity denote which antennal responses are significantly different from their respective control responses (Fisher's protected LSD; \* $P < 0.05$ , \*\* $P < 0.0001$ ).

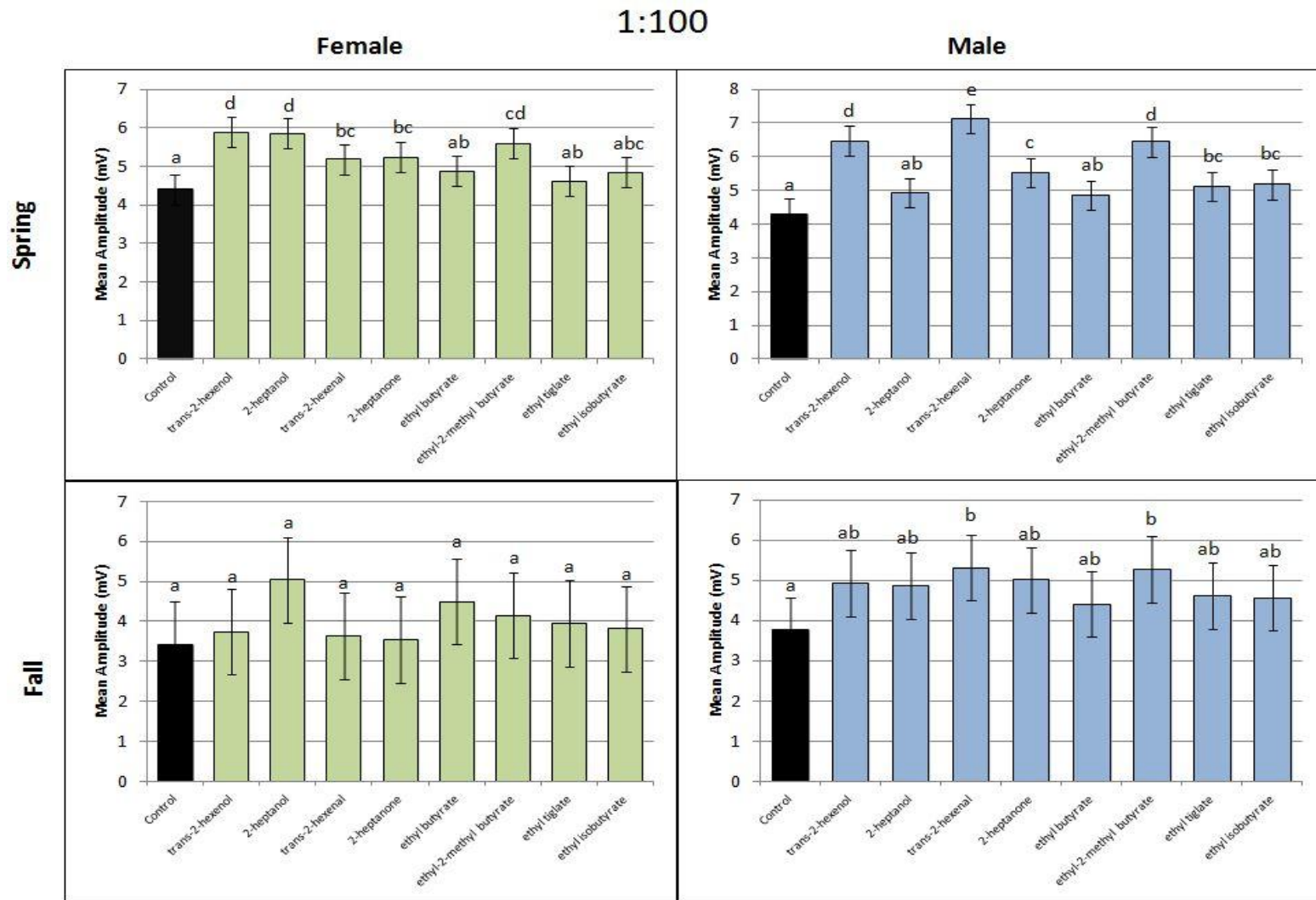


**Figure 11.** The mean ( $\pm$ SE) antennal responses of each group of *C. sayi* at the 1:10,000 dose for each compound. EAG amplitude means followed by the same letter are not significantly different (Fisher's protected LSD;  $P < 0.05$ ).

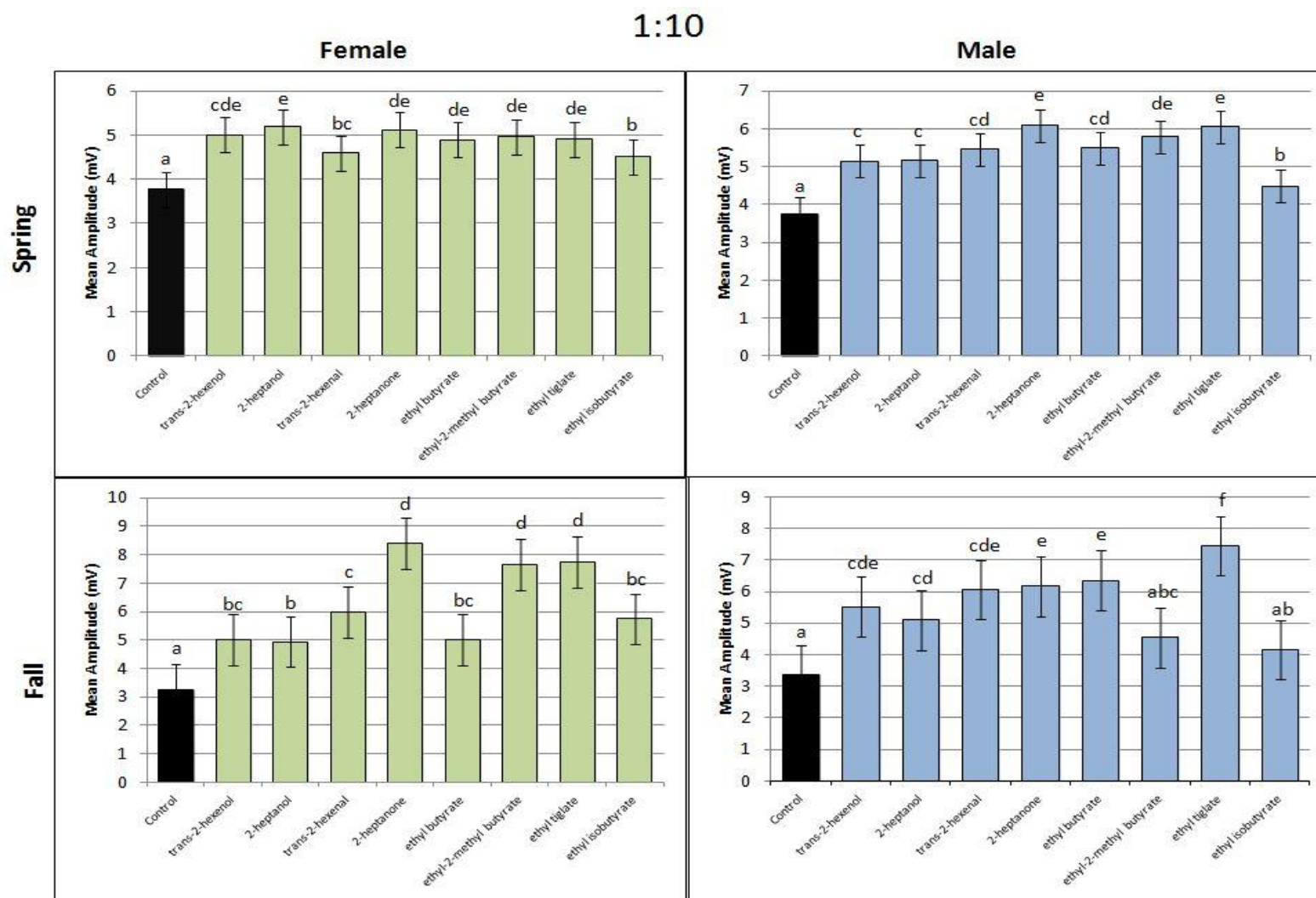




**Figure 12.** The mean ( $\pm$ SE) antennal responses of each group of *C. sayi* at the 1:1,000 dose for each compound. EAG amplitude means followed by the same letter are not significantly different (Fisher's protected LSD;  $P < 0.05$ ).



**Figure 13.** The mean ( $\pm$ SE) antennal responses of each group of *C. sayi* at the 1:100 dose for each compound. EAG amplitude means followed by the same letter are not significantly different (Fisher's protected LSD;  $P < 0.05$ ).



**Figure 14.** The mean ( $\pm$ SE) antennal responses of each group of *C. sayi* at the 1:10 dose for each compound. EAG amplitude means followed by the same letter are not significantly different (Fisher's protected LSD;  $P < 0.05$ ).

## CHAPTER III:

### EVALUATING CHESTNUT WEEVIL BEHAVIORAL RESPONSES TO DIFFERENT DOSES OF HOST PLANT VOLATILE ORGANIC COMPOUNDS (VOC) USING Y-TUBE OLFACTOMETRY

#### Introduction

*Curculio sayi* (Gyllenhal), the lesser (or small) chestnut weevil, is a key economic pest of chestnut in the central and eastern regions of the United States (Brooks and Cotton, 1929). The host plants of this native weevil species are limited to only members of the genus *Castanea* (which include chestnut and chinquapin). *C. sayi* is reported to have a 2-3 year life cycle with populations having two annual periods of activity (Brooks and Cotton 1929, Johnson 1956, Keesey and Barrett 2008). The first activity period occurs in the spring when chestnut catkins are blooming. During this time the adult weevils emerge from the ground and move into the trees and feed on the catkins. After catkin senescence the weevils leave the tree and, presumably, return to the ground debris where they enter a period of inactivity (Anagnostakis 2005).

The second period of adult activity occurs during late-summer/early-fall when the chestnut burs begin to split. The adult weevils that had been resting in the duff on the ground (and other secluded sites) become active again and return to the chestnut tree canopy. Additionally, Keesey and Barrett (2008) reported that during this period in the fall a second (but smaller) emergence of adults from the soil also occurs. After mating the female weevil begins to lay eggs (usually in September) by chewing a hole through the nut and sometimes the bur. The developing larvae will feed on the nut contents for about 3 weeks, after which it will emerge from the nut and burrow into the soil to pupate (Brooks and Cotton 1929, Johnson 1956).

Rodriguez-Saona and Stelinski (2009) suggested that an understanding of the behavioral responses of the target pest to its host plant volatile organic compounds (VOCs) must be known before such VOCs can be effectively utilized in pest management programs (such as a monitoring trap attractant). Previous research identified eight chestnut volatile organic compounds (VOCs) as being potentially attractive to *C. sayi* (Keeseey 2013, Keeseey and Barrett 2012). The purpose of this study was to evaluate the level of behavioral activity of *C. sayi* towards these key host plant VOCs using Y-tube olfactometry, at various doses, and to compare the behavioral responses across weevil sex and season of adult activity.

## **Materials and Methods**

### **Field Site and Weevil Collection**

Adult *Curculio sayi* were collected from a private farm near the city of Glasgow within Saline County (39.19190° N, 292.93110° W), Missouri. Planted on the farm were several different nut trees including several different chestnuts (*Castanea spp.*). The USDA soil type at the farm was Menfro silt loam. The site contains 14 chestnuts of different varieties spaced between 7 to 10 meters apart with overlapping canopies. The trees are 15-18 m tall and were estimated between 40 and 50 years of age and a grafted variety cross of Asian and American chestnut species (Ken Hunt, correspondence). The tree's catkins grow through April to June and nuts begin to drop in August continuing all the way through October.

*C. sayi* were collected using three types of traps: ground-based emergence traps, tree-mounted circle traps and silhouette traps (for detailed descriptions of trap specifics see Keeseey 2008). Additionally, a limb-tapping technique with canvas drop cloths to

catch falling weevils dislodged from the canopy was employed. Once collected weevils were sexed using proboscis length and shape (a sexually dimorphic trait) and then separated for transport back to the laboratory. In the laboratory the weevils were stored in plastic half liter cups containing 2-3 cm bedding of pine wood shavings. Two sponge cubes saturated with honey water were kept in each cup, which contained about 12 weevils (same sex). Cups were stored in a growth chamber set with a 14:10 (L:D) hour photoperiod and a temperature of 27° C. Collected weevils were tested only during the same period as their emergence, i.e. no spring weevils were tested or utilized during the fall testing period.

### **Y-tube Olfactometry**

The Y-tube olfactometer consisted of glassware of two 10 cm arms connected to a 15 cm stem (24 mm diameter) (Analytical Research Systems, Gainesville, FL) (Figure 15). Compressed air was humidified and filtered with active charcoal then passed through two inline flow meters. The flow meters controlled the airflow through either arm of the Y-tube at a rate of 0.5 liter/min. Glass holding chambers (15 cm in length by 3 cm in diameter) were used to introduce 1 µL treatment and control odors on filter paper wedges (Whatman No.4) into the air flow with connections made with Teflon tubing. The assembly was centered about 3 m beneath a fluorescent light fixture containing two 1 m long 32-watt bulbs producing between 310 and 340 lux. The Y-tube was held at a 30° angle in a white-walled cardboard enclosure to prevent the interference of visual cues.

Individual weevils were introduced to the Y-tube using a glass release chamber connected to the Y-tube stem. A choice was recorded when an insect traveled up the stem and into the end of either of the arms of the Y-tube. If within 5 minutes the insect

did not reach the end of one of the Y-tube arms, the weevil was removed and a 'no choice' was recorded. There were at least 10 replications per treatment with weevils being tested twice at both positions of the odor source (the Y-tube was flipped to prevent any directional bias). Tested *C. sayi* fasted for 24 hours prior and given a recovery period of at least 24 hours after testing. Due to the limited number of weevils, previously tested weevils were reused for further repetitions (the 48 hour periods between tests acted to negate any past interference). All Y-tube glassware was cleaned with hot soapy water and rinsed with methanol and acetone before being left to air-dry overnight.

### **Solution Preparation and Data Analysis**

The chestnut VOCs that were evaluated were: (E)-2-hexenol, 2-heptanol, (E)-2-hexenal, 2-heptanone, ethyl butyrate, ethyl-2-methyl butyrate, ethyl tiglate, and ethyl isobutyrate. Treatment solutions were prepared at four different dilutions: 1:10, 1:100, 1:1,000, and 1:10,000 compound to solvent, based on the responsiveness of individual volatiles to the solvent laboratory grade mineral oil (Sigma Aldrich, St. Louis, MO). The purity of each of the synthetic VOCs was high (over 95%). Fresh treatment solutions were prepared each day for weevils to be tested.

The data were analyzed with a logistic analysis of variance (ANOVA) (PROC GENMOD; SAS v. 9.3, SAS Institute Inc., Cary, NC) using a logit link and a binomial distribution. The explanatory variables examined were compound, dose, sex of the weevil, and season of weevil activity. Treatments were arranged as an 8 x 4 x 2 x 2 factorial (compound, dose, sex and season, respectively). Least-squares means (PROC GENMOD; LSMEANS; SAS v. 9.3) were used to test mean differences.

### **Results**

Y-tube bioassay responses from female and male weevils collected during the spring and fall emergence periods (2012) were analyzed individually by dose and sex with comparisons of the compounds. Overall, there was a high level of responsiveness (either choosing the control arm or the treatment arm of the Y-tube olfactometer) with only 14% of weevils not making a choice. Consideration of all variables simultaneously was not reported due to low repetitions (around n=10 for each combination), although dose and compound were maintained throughout so they could be directly examined.

The analysis of variance showed no significant effects when compound, dose, sex and season were considered independently ( $P < 0.05$ ) (Table 2). Considering all the possible two-way interactions, only the interaction between sex and season was significant with a P value of 0.037. The three-way and four-way interactions were not significant.

### **Responses to (E)-2-hexenol**

At the 1:10,000 dilution of (E)-2-hexenol, spring weevils (sexes combined) responded by choosing the control treatment 10 times and the VOC treatment 5 times (Figure 16), while the fall active weevils chose the control treatment 9 times and the VOC treatment 10 times (Figure 17). At the 1:1,000 dilution, spring active weevils responded with 8 control choices of the control treatment and 9 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 13 times and the VOC treatment 7 times (Figure 17).

At the 1:100 dilution of (E)-2-hexenol, spring weevils (sexes combined) responded by choosing the control treatment 10 times and the VOC treatment 8 times (Figure 16), while the fall active weevils chose the control treatment 9 times and the



VOC treatment 10 times (Figure 17). At the 1:10 dilution, spring active weevils responded with 12 control choices of the control treatment and 6 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 10 times and the VOC treatment 8 times (Figure 17). There were no significant differences between control and treatment responses at any dose for weevils of either period of emergence.

When considering just dose and weevil sex (weevil periods of activity combined) towards (E)-2-hexenol, adult *C. sayi* responded to each dose as follows. At the 1:10,000 dilution, male weevils responded with 10 control choices and 7 treatment choices (Figure 18), while female weevils responded with 9 control choices and 8 treatment choices (Figure 19). At the 1:1,000 dilution, male weevils responded with 11 control choices and 7 treatment choices (Figure 18), and female weevils responded with 10 control choices and 9 treatment choices (Figure 19).

At the 1:100 dilution of (E)-2-hexenol, the male weevils chose the control treatment 10 times and the VOC treatment 9 times (Figure 18), and the females chose the control 9 times and the VOC treatment 9 times (Figure 19). At the 1:10 dilution, male weevils choose the control treatment 8 times and the VOC treatment 10 times (Figure 18), while female weevils choose control 10 times and treatment 9 times (Figure 19). Female weevils responded significantly to (E)-2-hexenol at the 1:10 dilution and there were no other significant differences between control and treatment responses at any dose for weevils of either sex.

When considering only the dose of (E)-2-hexenol (weevil sex and period of seasonal activity), at the 1:10,000 dilution the weevils selected the control treatment 19 times and

the VOC treatment 15 times (Figure 20). At the 1:1,000 dilution, the weevils responded with 21 control choices and 16 treatment choices. At the 1:100 dilution, the control treatment was selected 19 control times and the VOC treatment 18 times. At the 1:10 dilution, the weevils choose the control 22 times and treatment 14 times (Figure 20). There were no significant differences between control and treatment responses at any dilution dose.

### **Responses to 2-heptanol**

At the 1:10,000 dilution of 2-heptanol, spring weevils (sexes combined) responded by choosing the control treatment 10 times and the VOC treatment 6 times (Figure 16), while the fall active weevils chose the control treatment 8 times and the VOC treatment 7 times (Figure 17). At the 1:1,000 dilution, spring active weevils responded with 20 control choices of the control treatment and 8 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 4 times and the VOC treatment 13 times (Figure 17).

At the 1:100 dilution of 2-heptanol, spring weevils (sexes combined) responded by choosing the control treatment 8 times and the VOC treatment 12 times (Figure 16), while the fall active weevils chose the control treatment 5 times and the VOC treatment 10 times (Figure 17). At the 1:10 dilution, spring active weevils responded with 11 control choices of the control treatment and 4 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 7 times and the VOC treatment 10 times (Figure 17). Spring-active weevils responded significantly to 2-heptanol at the 1:1,000 dilution and there were no other significant differences between control and treatment responses at any dose for weevils of either period of emergence.

When considering just dose and weevil sex (weevil periods of activity combined) towards 2-heptanol, adult *C. sayi* responded to each dose as follows. At the 1:10,000 dilution, male weevils responded with 7 control choices and 7 treatment choices (Figure 18), while female weevils responded with 7 control choices and 10 treatment choices (Figure 19). At the 1:1,000 dilution, male weevils responded with 17 control choices and 11 treatment choices (Figure 18), and female weevils responded with 7 control choices and 10 treatment choices (Figure 19).

At the 1:100 dilution of 2-heptanol, the male weevils chose the control treatment 8 times and the VOC treatment 5 times (Figure 18), and the females chose the control 10 times and the VOC treatment 9 times (Figure 19). At the 1:10 dilution, male weevils choose the control treatment 8 times and the VOC treatment 10 times (Figure 18), while female weevils choose control 10 times and treatment 9 times (Figure 19). There were no significant differences between control and treatment responses at any dose for weevils of either sex.

When considering only the dose of 2-heptanol (weevil sex and period of seasonal activity), at the 1:10,000 dilution the weevils selected the control treatment 16 times and the VOC treatment 19 times (Figure 20). At the 1:1,000 dilution, the weevils responded with 15 control choices and 14 treatment choices. At the 1:100 dilution, the control treatment was selected 19 control times and the VOC treatment 17 times. At the 1:10 dilution, the weevils choose the control 21 times and treatment 17 times (Figure 20). There were no significant differences between control and treatment responses at any dilution dose.

### **Responses to (E)-2-hexenal**

At the 1:10,000 dilution of (E)-2-hexenal, spring weevils (sexes combined) responded by choosing the control treatment 8 times and the VOC treatment 8 times (Figure 16), while the fall active weevils chose the control treatment 12 times and the VOC treatment 4 times (Figure 17). At the 1:1,000 dilution, spring active weevils responded with 10 control choices of the control treatment and 9 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 7 times and the VOC treatment 12 times (Figure 17).

At the 1:100 dilution of (E)-2-hexenal, spring weevils (sexes combined) responded by choosing the control treatment 12 times and the VOC treatment 6 times (Figure 16), while the fall active weevils chose the control treatment 10 times and the VOC treatment 8 times (Figure 17). At the 1:10 dilution, spring active weevils responded with 10 control choices of the control treatment and 10 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 4 times and the VOC treatment 12 times (Figure 17). There were no significant differences between control and treatment responses at any dose for weevils of either period of emergence.

When considering just dose and weevil sex (weevil periods of activity combined) towards (E)-2-hexenal, adult *C. sayi* responded to each dose as follows. At the 1:10,000 dilution, male weevils responded with 10 control choices and 7 treatment choices (Figure 18), while female weevils responded with 10 control choices and 5 treatment choices (Figure 19). At the 1:1,000 dilution, male weevils responded with 5 control choices and 13 treatment choices (Figure 18), and female weevils responded with 12 control choices and 7 treatment choices (Figure 19).

At the 1:100 dilution of (E)-2-hexenal, the male weevils chose the control treatment 10 times and the VOC treatment 8 times (Figure 18), and the females chose the control 12 times and the VOC treatment 6 times (Figure 19). At the 1:10 dilution, male weevils choose the control treatment 6 times and the VOC treatment 12 times (Figure 18), while female weevils choose control 8 times and treatment 10 times (Figure 19). Male weevils responded significantly to (E)-2-hexenal at the 1:1,000 dose and there were no other significant differences between control and treatment responses at any dose for weevils of either sex.

When considering only the dose of (E)-2-hexenal (weevil sex and period of seasonal activity), at the 1:10,000 dilution the weevils selected the control treatment 21 times and the VOC treatment 16 times (Figure 20). At the 1:1,000 dilution, the weevils responded with 24 control choices and 21 treatment choices. At the 1:100 dilution, the control treatment was selected 17 control times and the VOC treatment 21 times. At the 1:10 dilution, the weevils choose the control 27 times and treatment 18 times (Figure 20). There were no significant differences between control and treatment responses at any dilution dose.

### **Responses to 2-heptanone**

At the 1:10,000 dilution of 2-heptanone, spring weevils (sexes combined) responded by choosing the control treatment 15 times and the VOC treatment 5 times (Figure 16), while the fall active weevils chose the control treatment 9 times and the VOC treatment 8 times (Figure 17). At the 1:1,000 dilution, spring active weevils responded with 16 control choices of the control treatment and 14 treatment choices of

the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 11 times and the VOC treatment 4 times (Figure 17).

At the 1:100 dilution of 2-heptanone, spring weevils (sexes combined) responded by choosing the control treatment 18 times and the VOC treatment 16 times (Figure 16), while the fall active weevils chose the control treatment 10 times and the VOC treatment 5 times (Figure 17). At the 1:10 dilution, spring active weevils responded with 6 control choices of the control treatment and 9 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 9 times and the VOC treatment 11 times (Figure 17). Spring active weevils responded to 2-heptanone at the 1:10,000 dilution and there were no other significant differences between control and treatment responses at any dose for weevils of either period of emergence.

When considering just dose and weevil sex (weevil periods of activity combined) towards 2-heptanone, adult *C. sayi* responded to each dose as follows. At the 1:10,000 dilution, male weevils responded with 11 control choices and 9 treatment choices (Figure 18), while female weevils responded with 13 control choices and 4 treatment choices (Figure 19). At the 1:1,000 dilution, male weevils responded with 17 control choices and 11 treatment choices (Figure 18), and female weevils responded with 10 control choices and 7 treatment choices (Figure 19).

At the 1:100 dilution of 2-heptanone, the male weevils chose the control treatment 16 times and the VOC treatment 8 times (Figure 18), and the females chose the control 12 times and the VOC treatment 13 times (Figure 19). At the 1:10 dilution, male weevils choose the control treatment 9 times and the VOC treatment 9 times (Figure 18), while female weevils choose control 6 times and treatment 11 times (Figure 19). There were no

significant differences between control and treatment responses at any dose for weevils of either sex.

When considering only the dose of 2-heptanone (weevil sex and period of seasonal activity), at the 1:10,000 dilution the weevils selected the control treatment 18 times and the VOC treatment 13 times (Figure 20). At the 1:1,000 dilution, the weevils responded with 21 control choices and 12 treatment choices. At the 1:100 dilution, the control treatment was selected 29 control times and the VOC treatment 23 times. At the 1:10 dilution, the weevils choose the control 14 times and treatment 19 times (Figure 20). There were no significant differences between control and treatment responses at any dilution dose.

### **Responses to ethyl butyrate**

At the 1:10,000 dilution of ethyl butyrate, spring weevils (sexes combined) responded by choosing the control treatment 7 times and the VOC treatment 8 times (Figure 16), while the fall active weevils chose the control treatment 9 times and the VOC treatment 11 times (Figure 17). At the 1:1,000 dilution, spring active weevils responded with 11 control choices of the control treatment and 5 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 7 times and the VOC treatment 8 times (Figure 17).

At the 1:100 dilution of ethyl butyrate, spring weevils (sexes combined) responded by choosing the control treatment 8 times and the VOC treatment 8 times (Figure 16), while the fall active weevils chose the control treatment 8 times and the VOC treatment 10 times (Figure 17). At the 1:10 dilution, spring active weevils responded with 7 control choices of the control treatment and 11 treatment choices of the

VOC treatment (Figure 16), and the fall active weevils chose the control treatment 9 times and the VOC treatment 11 times (Figure 17). There were no significant differences between control and treatment responses at any dose for weevils of either period of emergence.

When considering just dose and weevil sex (weevil periods of activity combined) towards ethyl butyrate, adult *C. sayi* responded to each dose as follows. At the 1:10,000 dilution, male weevils responded with 9 control choices and 9 treatment choices (Figure 18), while female weevils responded with 7 control choices and 10 treatment choices (Figure 19). At the 1:1,000 dilution, male weevils responded with 11 control choices and 5 treatment choices (Figure 18), and female weevils responded with 7 control choices and 8 treatment choices (Figure 19).

At the 1:100 dilution of ethyl butyrate, the male weevils chose the control treatment 5 times and the VOC treatment 10 times (Figure 18), and the females chose the control 11 times and the VOC treatment 8 times (Figure 19). At the 1:10 dilution, male weevils choose the control treatment 8 times and the VOC treatment 11 times (Figure 18), while female weevils choose control 8 times and treatment 11 times (Figure 19). There were no significant differences between control and treatment responses at any dose for weevils of either sex.

When considering only the dose of ethyl butyrate (weevil sex and period of seasonal activity), at the 1:10,000 dilution the weevils selected the control treatment 19 times and the VOC treatment 18 times (Figure 20). At the 1:1,000 dilution, the weevils responded with 13 control choices and 22 treatment choices. At the 1:100 dilution, the control treatment was selected 22 control times and the VOC treatment 14 times. At the 1:10



dilution, the weevils choose the control 28 times and treatment 21 times (Figure 20). There were no significant differences between control and treatment responses at any dilution dose.

### **Responses to ethyl-2-methyl butyrate**

At the 1:10,000 dilution of ethyl-2-methyl butyrate, spring weevils (sexes combined) responded by choosing the control treatment 8 times and the VOC treatment 8 times (Figure 16), while the fall active weevils chose the control treatment 7 times and the VOC treatment 6 times (Figure 17). At the 1:1,000 dilution, spring active weevils responded with 12 control choices of the control treatment and 3 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 9 times and the VOC treatment 9 times (Figure 17).

At the 1:100 dilution of ethyl-2-methyl butyrate, spring weevils (sexes combined) responded by choosing the control treatment 10 times and the VOC treatment 9 times (Figure 16), while the fall active weevils chose the control treatment 7 times and the VOC treatment 10 times (Figure 17). At the 1:10 dilution, spring active weevils responded with 11 control choices of the control treatment and 6 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 10 times and the VOC treatment 6 times (Figure 17). Spring active weevils responded significantly to ethyl-2-methyl butyrate at the 1:1,000 dilution and there were no other significant differences between control and treatment responses at any dose for weevils of either period of emergence.

When considering just dose and weevil sex (weevil periods of activity combined) towards ethyl-2-methyl butyrate, adult *C. sayi* responded to each dose as follows. At the

1:10,000 dilution, male weevils responded with 5 control choices and 7 treatment choices (Figure 18), while female weevils responded with 10 control choices and 7 treatment choices (Figure 19). At the 1:1,000 dilution, male weevils responded with 10 control choices and 3 treatment choices (Figure 18), and female weevils responded with 11 control choices and 9 treatment choices (Figure 19).

At the 1:100 dilution of ethyl-2-methyl butyrate, the male weevils chose the control treatment 8 times and the VOC treatment 9 times (Figure 18), and the females chose the control 9 times and the VOC treatment 10 times (Figure 19). At the 1:10 dilution, male weevils choose the control treatment 9 times and the VOC treatment 7 times (Figure 18), while female weevils choose control 12 times and treatment 5 times (Figure 19). There were no significant differences between control and treatment responses at any dose for weevils of either sex.

When considering only the dose of ethyl-2-methyl butyrate (weevil sex and period of seasonal activity), at the 1:10,000 dilution the weevils selected the control treatment 16 times and the VOC treatment 18 times (Figure 20). At the 1:1,000 dilution, the weevils responded with 17 control choices and 19 treatment choices. At the 1:100 dilution, the control treatment was selected 26 control times and the VOC treatment 30 times. At the 1:10 dilution, the weevils choose the control 16 times and treatment 21 times (Figure 20). There were no significant differences between control and treatment responses at any dilution dose.

### **Responses to ethyl tiglate**

At the 1:10,000 dilution of ethyl tiglate, spring weevils (sexes combined) responded by choosing the control treatment 8 times and the VOC treatment 11 times

(Figure 16), while the fall active weevils chose the control treatment 11 times and the VOC treatment 6 times (Figure 17). At the 1:1,000 dilution, spring active weevils responded with 18 control choices of the control treatment and 18 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 11 times and the VOC treatment 5 times (Figure 17).

At the 1:100 dilution of ethyl tiglate, spring weevils (sexes combined) responded by choosing the control treatment 18 times and the VOC treatment 18 times (Figure 16), while the fall active weevils chose the control treatment 8 times and the VOC treatment 12 times (Figure 17). At the 1:10 dilution, spring active weevils responded with 14 control choices of the control treatment and 18 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 7 times and the VOC treatment 12 times (Figure 17). There were no significant differences between control and treatment responses at any dose for weevils of either period of emergence.

When considering just dose and weevil sex (weevil periods of activity combined) towards ethyl tiglate, adult *C. sayi* responded to each dose as follows. At the 1:10,000 dilution, male weevils responded with 10 control choices and 7 treatment choices (Figure 18), while female weevils responded with 9 control choices and 10 treatment choices (Figure 19). At the 1:1,000 dilution, male weevils responded with 16 control choices and 11 treatment choices (Figure 18), and female weevils responded with 13 control choices and 12 treatment choices (Figure 19).

At the 1:100 dilution of ethyl tiglate, the male weevils chose the control treatment 11 times and the VOC treatment 12 times (Figure 18), and the females chose the control 15 times and the VOC treatment 18 times (Figure 19). At the 1:10 dilution, male weevils

choose the control treatment 10 times and the VOC treatment 15 times (Figure 18), while female weevils choose control 11 times and treatment 15 times (Figure 19). There were no significant differences between control and treatment responses at any dose for weevils of either sex.

When considering only the dose of ethyl tiglate (weevil sex and period of seasonal activity), at the 1:10,000 dilution the weevils selected the control treatment 22 times and the VOC treatment 14 times (Figure 20). At the 1:1,000 dilution, the weevils responded with 18 control choices and 14 treatment choices. At the 1:100 dilution, the control treatment was selected 14 control times and the VOC treatment 22 times. At the 1:10 dilution, the weevils choose the control 15 times and treatment 20 times (Figure 20). There were no significant differences between control and treatment responses at any dilution dose.

### **Responses to ethyl isobutyrate**

At the 1:10,000 dilution of ethyl isobutyrate, spring weevils (sexes combined) responded by choosing the control treatment 10 times and the VOC treatment 9 times (Figure 16), while the fall active weevils chose the control treatment 11 times and the VOC treatment 8 times (Figure 17). At the 1:1,000 dilution, spring active weevils responded with 7 control choices of the control treatment and 10 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 7 times and the VOC treatment 9 times (Figure 17).

At the 1:100 dilution of ethyl isobutyrate, spring weevils (sexes combined) responded by choosing the control treatment 10 times and the VOC treatment 9 times (Figure 16), while the fall active weevils chose the control treatment 6 times and the

VOC treatment 12 times (Figure 17). At the 1:10 dilution, spring active weevils responded with 6 control choices of the control treatment and 13 treatment choices of the VOC treatment (Figure 16), and the fall active weevils chose the control treatment 8 times and the VOC treatment 6 times (Figure 17). There were no significant differences between control and treatment responses at any dose for weevils of either period of emergence.

When considering just dose and weevil sex (weevil periods of activity combined) towards ethyl isobutyrate, adult *C. sayi* responded to each dose as follows. At the 1:10,000 dilution, male weevils responded with 10 control choices and 9 treatment choices (Figure 18), while female weevils responded with 11 control choices and 8 treatment choices (Figure 19). At the 1:1,000 dilution, male weevils responded with 7 control choices and 8 treatment choices (Figure 18), and female weevils responded with 7 control choices and 11 treatment choices (Figure 19).

At the 1:100 dilution of ethyl isobutyrate, the male weevils chose the control treatment 9 times and the VOC treatment 10 times (Figure 18), and the females chose the control 7 times and the VOC treatment 11 times (Figure 19). At the 1:10 dilution, male weevils choose the control treatment 10 times and the VOC treatment 10 times (Figure 18), while female weevils choose control 4 times and treatment 9 times (Figure 19). There were no significant differences between control and treatment responses at any dose for weevils of either sex.

When considering only the dose of ethyl isobutyrate (weevil sex and period of seasonal activity), at the 1:10,000 dilution the weevils selected the control treatment 16 times and the VOC treatment 22 times (Figure 20). At the 1:1,000 dilution, the weevils

responded with 21 control choices and 12 treatment choices. At the 1:100 dilution, the control treatment was selected 21 control times and the VOC treatment 30 times. At the 1:10 dilution, the weevils choose the control 14 times and treatment 19 times (Figure 20). There were no significant differences between control and treatment responses at any dilution dose.

### **Discussion**

Adult *Curculio sayi* responded significantly to several VOCs at different doses but without the expected increase in responsiveness as dose increased. The significant responses were spread among several compounds: (E)-2-hexenol, 2-heptanol, (E)-2-hexenal, 2-heptanone, and ethyl-2-methyl butyrate. Additionally the responses were spread across all four doses and combinations of weevils except for fall weevils and those disregarding both sex and season. The inconsistent spread of significant results among different groups of weevils suggests that chance may have played a role in which compounds/doses the weevils responded significantly to. This seems especially likely for responses to compounds at the lowest two doses, which were not responded to physiologically by weevils (Chapter II). A further consequence of the limited number of weevils and repetitions was the inability to examine interactions between sex and season. The response of females at the 1:10 dilution of (E)-2-hexenol was the only significant response that clearly agreed with the EAG results (Chapter II). Aside from just being significant the response to (E)-2-hexenol was slightly repellent suggesting that while it is behaviorally relevant other factors may be necessary if it is an attractant.

The rest of the responses that were not significant provide their own clues. First it is not likely that any of the compounds were tested at a dose where it was either

extremely attractive or repellent. If a compound was extremely attractive or repellent it would show up even with a fairly small sample size. This agrees with the conclusion from chapter II that the 1:10 dilution did not overload weevil receptors and therefore should not elicit repellent behavior in weevils trying to avoid being overwhelmed by an odor. Also it suggests that strong attraction may be achieved through synergistic effects between multiple VOCs as has been the case with other insects (Bruce et. al 2005, Piñero et al. 2001). Considering these results and those from chapter II, further work to identify an attractant should focus on concentrations similar to the 1:10 and 1:100 dilutions or higher since weevils only appeared to consistently detect most of the volatiles at 1:100 and were not clearly repelled at the highest dose. Also testing compound mixtures could identify any synergistic effects responsible for attraction.

Another alternative may be to use mixtures of promising plant VOCs with pheromones, a technique that has enhanced attraction in the boll weevil, *Anthonomus grandis* Boheman (Dickens 1989) and the plum curculio, *Conotrachelus nenuphar* (Piñero et al. 2001). Unfortunately for *C. sayi* a sex or aggregation pheromone has not been identified to date. However, the attraction of *C. sayi* to plant tissue found by Keesey (2011), particularly to the catkins among male spring-active weevils and female weevils of both periods of activity, suggests that plant odors alone can produce attraction without pheromones. Additionally, the lack of significant behavioral responses to both bur and nut tissue (aside from the attraction of fall females to bur tissue in the fall) agrees with the lack of behavior responses from esters (ethyl butyrate, ethyl tiglate, etc.) in our results as these VOCs were the ones produced by nut and bur tissue. In large part these esters were selected because when tested individually they received repellent responses

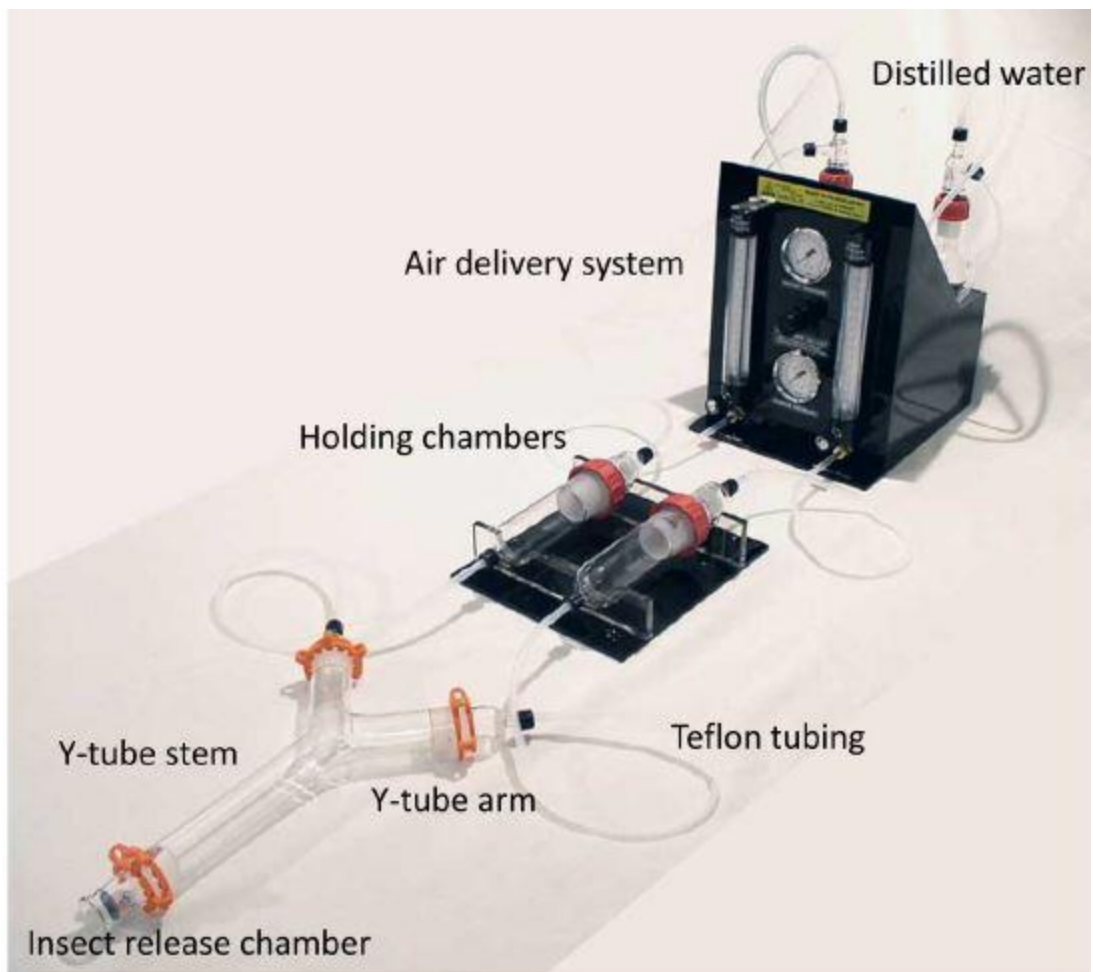
from weevils. Often repellent responses, as in the case of the granary weevil, *Sitophilus granarius* (L.), from a specific volatile may vary with the dose or even if they do not may be part of the different positive and negative stimuli regulating weevil behavior (Germinara et al. 2008). Combinations of VOCs should provide more information about not only how these compounds individually effect weevils but could be effect in an environment with other chestnut odors present.



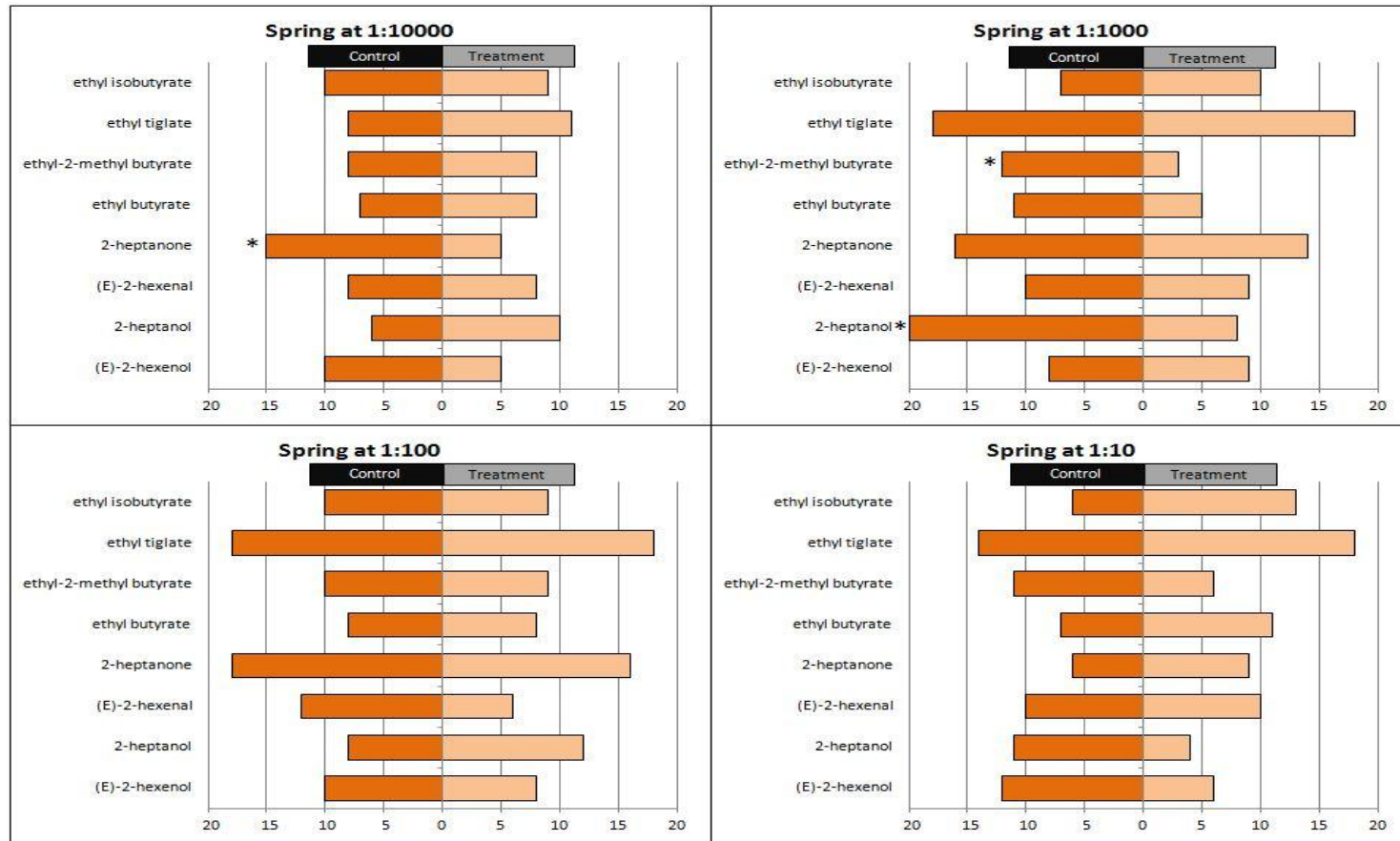
### Y-tube Bioassay Results

Effect	Num DF	Chi-square	Pr > Chi Sq
<b>Compound</b>	10	24.20	0.1999
<b>Dose</b>	1	1.11	0.1975
<b>Sex</b>	1	5.25	0.9554
<b>Season</b>	10	23.13	0.1412
Compound*Dose	10	6.47	0.3135
Compound*Sex	10	0.00	0.5409
Dose*Sex	1	3.75	0.7193
Compound*Dose*Sex	10	3.75	0.5124
Compound*Season	10	3.75	0.4327
Dose*Season	10	3.75	0.5399
Compound*Dose*Season	10	3.75	0.1503
Sex*Season	10	3.75	0.0370
Compound*Sex*Season	10	3.75	0.1860
Dose*Sex*Season	10	3.75	0.2492
Compound*Dose*Sex* Season	10	3.75	0.1837

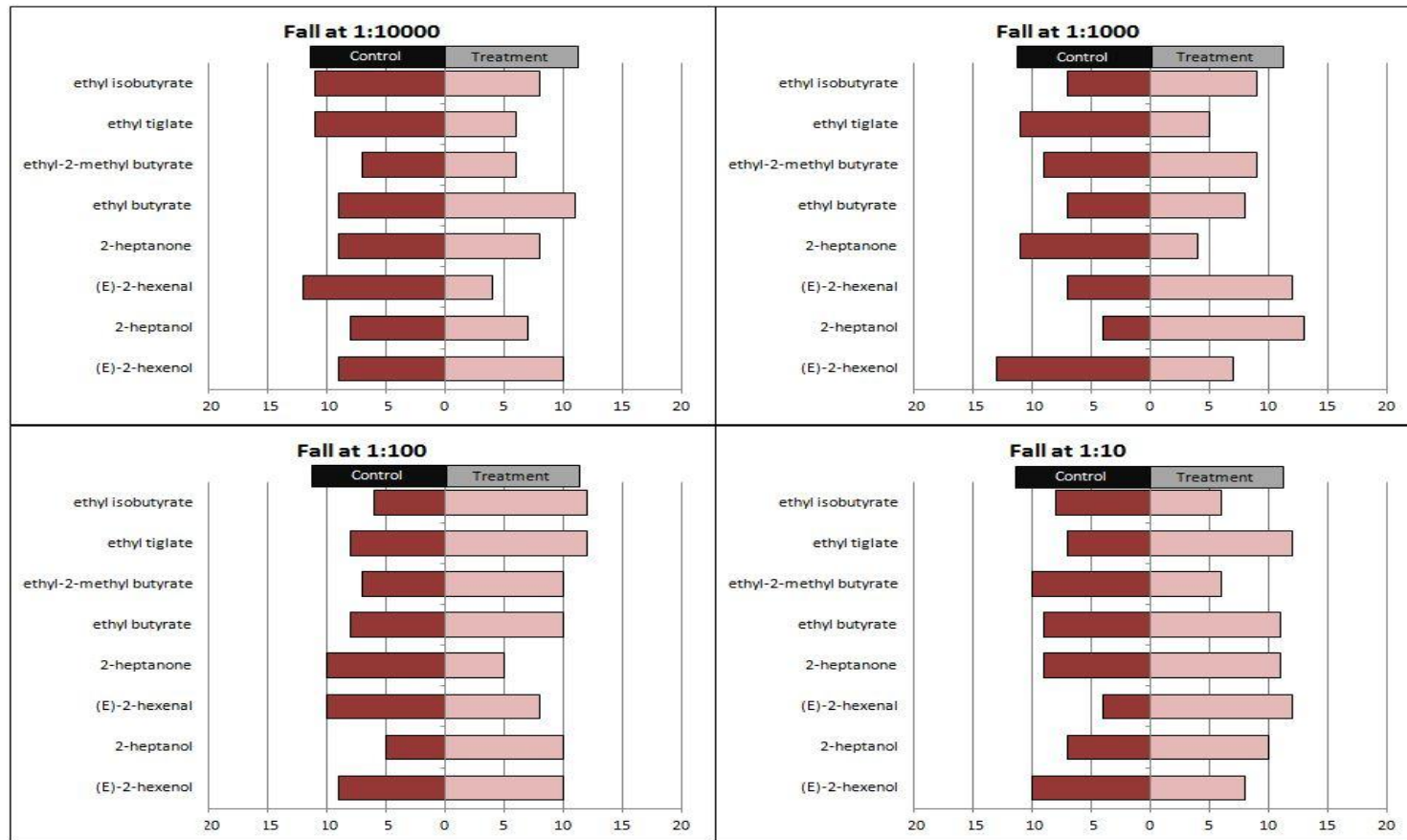
**Table 2.** Results of ANOVA performed on treatment compound, dose of compound, beetle sex, and season of beetle activity data from Y-tube bioassays.



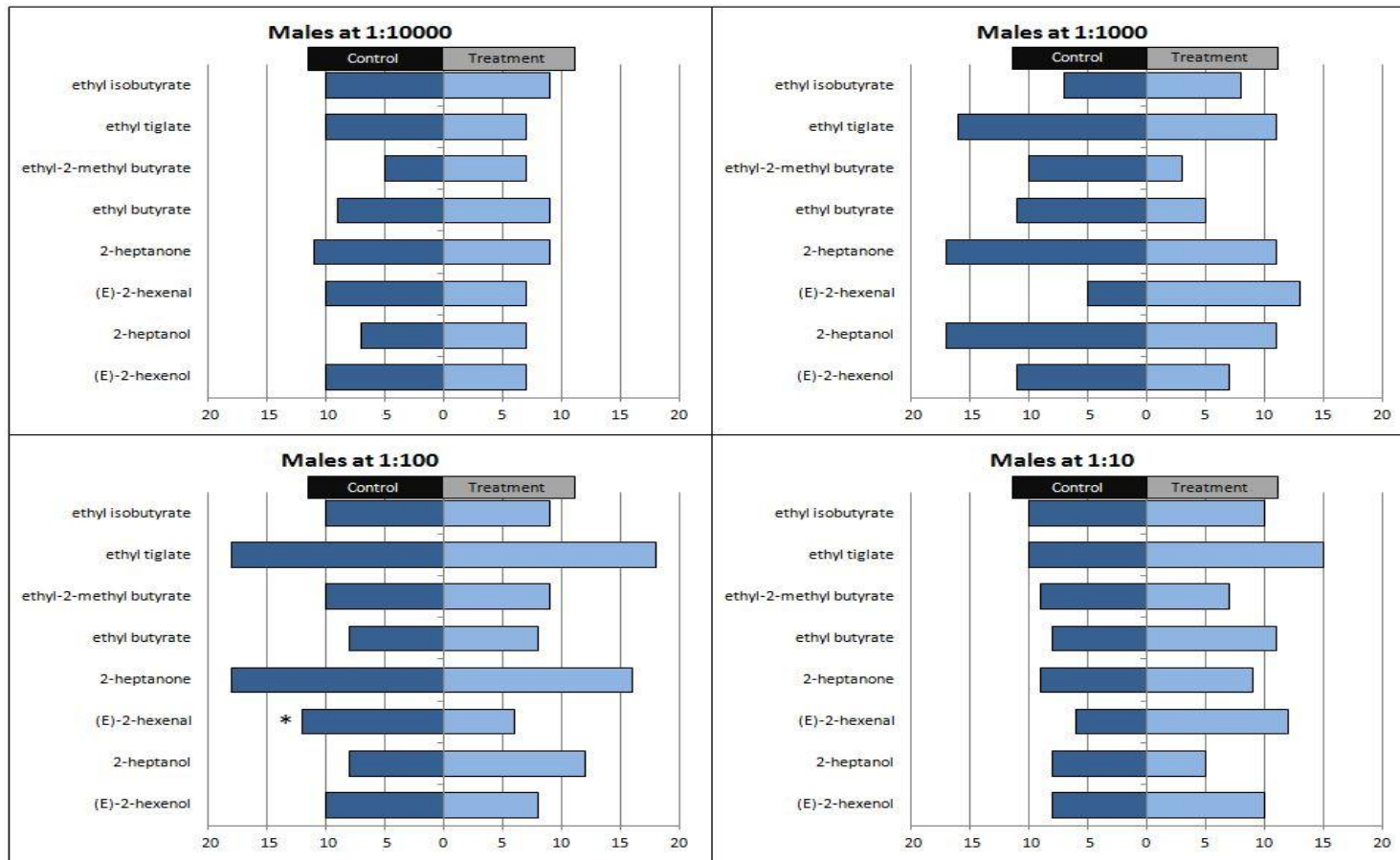
**Figure 15.** Y-tube olfactometer including air filtering and flow controllers along with treatment release chamber (image from Analytical Research Systems, Inc., Gainesville, FL).



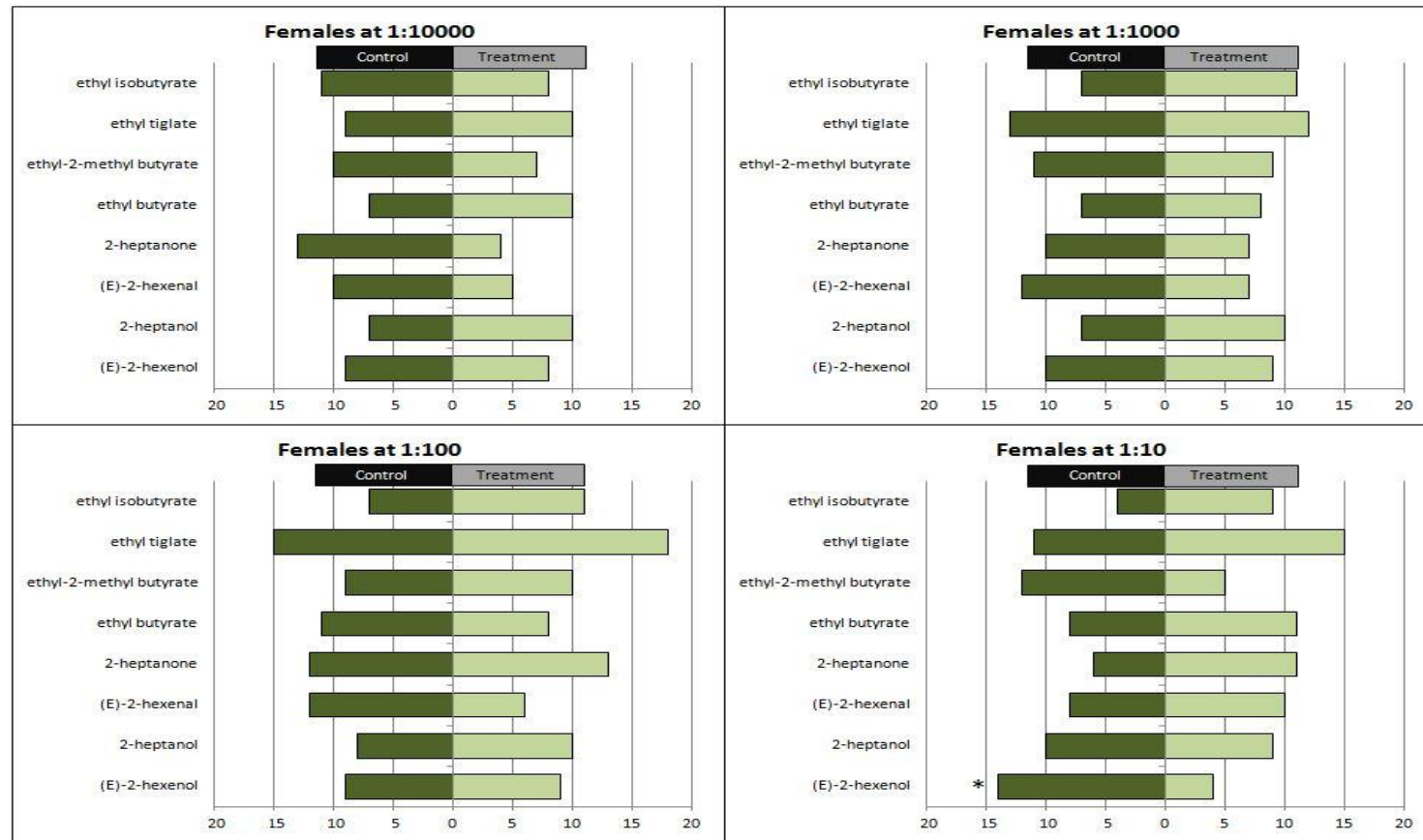
**Figure 16.** The behavioral responses (choices between a control and treatment VOC) of spring active adult *C. sayi* in a Y-tube olfactometer to selected VOCs at four dilutions (weevil sex data combined). Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



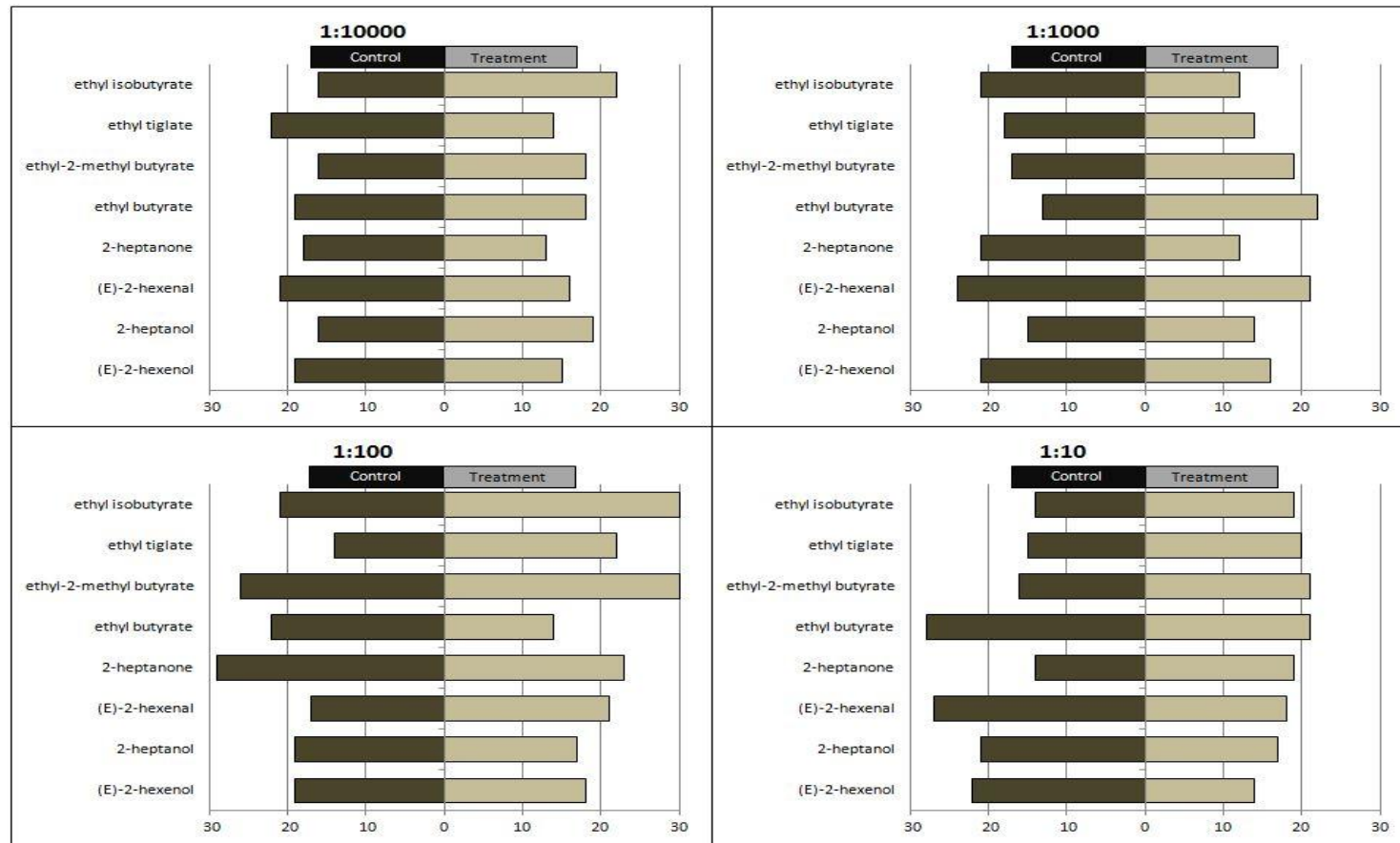
**Figure 17.** The behavioral responses (choices between a control and treatment VOC) of fall active adult *C. sayi* in a Y-tube olfactometer to selected VOCs at four dilutions (weevil sex data combined). Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



**Figure 18.** The behavioral responses (choices between a control and treatment VOC) of male adult *C. sayi* in a Y-tube olfactometer to selected VOCs at four dilutions (seasonal period of weevil activity combined). Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



**Figure 19.** The behavioral responses (choices between a control and treatment VOC) of female adult *C. sayi* in a Y-tube olfactometer to selected VOCs at four dilutions (seasonal period of weevil activity combined). Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



**Figure 20.** The behavioral responses (choices between a control and treatment VOC) of adult *C. sayi* in a Y-tube olfactometer to selected VOCs at four dilutions (weevil sex and seasonal period of activity combined). Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.

## CHAPTER IV:

### EVALUATING CHESTNUT WEEVIL ELECTROANTENNOGRAM (EAG) RESPONSES TO SINGLE DOSE MIXTURES OF HOST PLANT VOLATILE ORGANIC COMPOUNDS (VOC)

#### Introduction

The lesser (or small) chestnut weevil, *Curculio sayi* Gyllenhal is a host specific chestnut pest that lays its eggs in nuts. The developing larvae feed on the nut contents and emerge to pupate in the soil. *C. sayi* is a critical pest in the growing American chestnut market and can infest over 90% of nuts if left unchecked (Brooks and Cotton 1929). Methods of control have focused on post-harvest treatments and general pesticide use (Payne et al. 1983, Hunt et al. 2009, Brooks and Cotton 1929). Recently however the focus has shifted to more specific and proactive strategies with a focus on understanding the plant-insect relationship. The discovery of an additional emergence period (Keeseey and Barrett 2008) along with the specific solutions developed for similar pest are likely responsible for this shift in strategy. In several other systems (Szendrei et al. 2009, Sun et al. 2010, Adesso et al. 2011), understanding and manipulating host plant volatiles has served as an effective method of control.

Host plant volatiles are made up of many component volatile organic compounds (VOCs) that insects are able to detect. Similar to pheromone detection, host plant detection often relies on multiple components and can have several synergists or antagonists (Hansson 2002). In order to both isolate the key VOCs and understand their importance in context two steps are generally used. First the individual VOCs are tested to determine which are detectable and at what concentration (Kozłowski and Visser 1981,



Gunawardena and Dissanayake 2000, Leskey et al. 2001). Then the selected VOCs are recombined to be tested for interactions (Piñero et al. 2001, van Tol et al. 2012).

Previous research has revealed several VOCs that are physiologically active for *C. sayi* (Keeseey and Barrett 2012, Keeseey et al. 2012). These volatile organic compounds include: 2-heptanol, (E)-2-hexenol, (E)-2-hexenal, ethyl tiglate, ethyl butyrate, ethyl isobutyrate, ethyl-2-methyl butyrate, and 2-heptanone (Keeseey and Barrett 2012). The purpose of this study was to determine the antennal sensitivity of *C. sayi* to mixtures of some of these key host plant VOCs using electroantennography (EAG), at a single dose, and to compare EAG responses across weevil sex and season of adult activity.

## **Materials and Methods**

### **Field Site and Weevil Collection**

Adult *Curculio sayi* were collected from a private farm near the city of Glasgow, Saline County (39.19190° N, 292.93110° W), Missouri. Planted on the farm were several different nut trees including several different chestnuts (*Castanea spp.*). The USDA soil type at the farm was Menfro silt loam. The site contains 14 chestnuts of different varieties spaced between 7 to 10 meters apart with overlapping canopies. The trees are 15-18 m tall and were estimated between 40 and 50 years of age and a grafted variety cross of Asian and American chestnut species (Ken Hunt, correspondence). The tree's catkins grow through April to June and nuts begin to drop in August continuing all the way through October.

*C. sayi* were collected using three types of traps: ground-based emergence traps, tree-mounted circle traps and silhouette traps (for detailed descriptions of traps see Keeseey 2008). Additionally, a limb-tapping technique with canvas drop cloths to catch

falling weevils dislodged from the canopy were employed. Once collected weevils were sexed using proboscis length and shape (a sexually dimorphic trait) and then separated for transport back to the laboratory. In the laboratory the weevils were stored in plastic half liter cups containing 2-3 cm bedding of pine wood shavings. Two sponge cubes saturated with honey water were kept in each cup that contained about 12 weevils (same sex). Cups were stored in a growth chamber set with a 14:10 (L:D) hour photoperiod and a temperature of 27° C. Collected weevils were tested only during the same period as their emergence, i.e. no spring weevils were tested or utilized during the fall testing period.

### **Antennal Preparation and EAG**

Antennal preparations and EAG procedures as described by Keeseey (2011) were followed. Such procedures that minimized baseline noise, provided maximum sensitivity and lowered preparation time. For example, weevil antennae were excised by pulling them out at an angle perpendicular to the head. This procedure allows for the removal of internal neural connections with the antennae. Both antenna flagella were then placed across a forked probe (Syntech, Netherlands) and partially immersed in Spectra 360 electrode gel (Parker Laboratories, Inc., Fairfield, New Jersey) that was used as a connection medium. The probe was attached to a high-impedance electrometer and an indifferent grounding electrode (EAG Kombi Probe, type PRG-3; Syntech, Hilversum, Netherlands). An "x-y-z" coordinate micromanipulator (MP-15; Syntech, Hilversum, Netherlands) on a magnetic base was used to position the antennal preparation into a constant humidified and charcoal-purified air stream (0.5 liters/min). The air stream was carried through a 10 mm glass tube that flanged to 15 mm to contain the VOC

preparation. An insertion point 13 cm down the glass tube away from the antennal preparation allowed odors to be delivered directly into the air stream using puff cartridges. Puff cartridges consisted of a 15 cm borosilicate glass Pasteur pipette 6 mm in diameter containing a 1 x 1 cm disc of filter paper (Whatman No. 4) injected with 1  $\mu$ L of treatment solution. New puff cartridges were prepared for each antennal preparation and individual treatment (Figure 21). Three puffs of air, 1.0 second in duration (100 ml/min), were delivered at 30 second intervals with at least one minute between treatments and the replacement of a new puff cartridge. A stimulus flow controller (CS-55; Syntech, Hilversum, Netherlands) was used to manage both the constant airflow and air puffs (Figure 22).

### **Volatile Organic Compounds and Data Analysis**

The chestnut VOCs that were evaluated were: (E)-2-hexenol (hereafter referred to as treatment A), (E)-2-hexenal (hereafter referred to as treatment B), 2-heptanone (hereafter referred to as treatment C), and ethyl butyrate (hereafter referred to as treatment D). Treatment solutions were prepared at a 1:10 dilution, compound to solvent, based on the responsiveness of individual volatiles to the solvent laboratory grade mineral oil (Sigma Aldrich, St. Louis, MO). In addition, mixtures consisting of two, three and four of the compounds were also prepared. Each treatment (and treatment mixture) was tested on the antennal preparation once with control puffs of solvent at the beginning of a recording, after the 6th stimulus puff and following the last mixture. Antennal responses were recorded using Syntech software (GC-EAD 2010) after they were passed through a high-impedance amplifier in a two-channel acquisition system optimized for EAG signals (IDAC-2; Syntech, Hilversum, Netherlands) (Figure 22).

Noise was controlled using a 150 kg steel plate base (3" x 12" x 18") placed on a 2 cm thick rubber pad as the attachment point for the antennal preparation. The sex and season of activity for adult weevils was recorded along with the antennal responses. Antennal response amplitude was measured as absolute value difference from the baseline.

The data were analyzed with a 3- way analysis of variance (ANOVA) using a split plot design (PROC MIXED; SAS v. 9.3, SAS Institute Inc., Cary, NC). The explanatory variables considered were the sex of the weevil, the season of weevil activity, and VOC mixture. The main plot consisted of sex, season, and sex by season while the subplot included mixture and all other possible interactions with the main plot effects. Mean differences were determined using least-squares means (PROC MIXED; LSMEANS; SAS v. 9.3) for comparisons between compound mixtures and weevils of either season of activity and sex.

## **Results**

The analysis of variance showed significant effects ( $P < 0.05$ ) individually from sex, season and mixture (Table 3). Considering all the possible two-way interactions, only the interaction of mixture and season had a significant effect. The three-way interaction between mixture, sex, and season was not significant.

### **Spring Weevil EAG Responses**

Electroantennogram responses from female weevils collected during the spring emergence period (2013) were analyzed individually by sex with comparisons of the compound-mixtures. Most treatment means were significantly greater than their control means at the  $P < 0.0001$  level, except treatment AB that was significantly greater at the  $P < 0.05$  level (Figure 23). Regarding treatment comparisons, treatment AB ((E)-2-hexenol

+ (E)-2-hexenal) generated the smallest mean EAG response (2.958 mV), significantly less than all the other treatment mixtures. Mean EAG responses to treatment mixtures ABD ((E)-2-hexenol + (E)-2-hexenal + ethyl butyrate) (4.273 mV), ABC ((E)-2-hexenol + (E)-2-hexenal + 2-heptanone) (4.549 mV), ABCD ((E)-2-hexenol + (E)-2-hexenal + 2-heptanone + ethyl butyrate) (4.738 mV), and BCD (E)-2-hexenal + 2-heptanone + ethyl butyrate) (5.200 mV) were not significantly different from each other, but they were significantly less than treatment mixtures ACD ((E)-2-hexenol + 2-heptanone + ethyl butyrate) (6.207 mV), AC ((E)-2-hexenol + 2-heptanone) (6.800 mV), AD ((E)-2-hexenol + ethyl butyrate) (7.915 mV), BD ((E)-2-hexenal + ethyl butyrate) (9.653 mV), BC ((E)-2-hexenal + 2-heptanone) (10.450 mV), and CD (2-heptanone + ethyl butyrate) (12.2479 mV). Treatments ACD and AC were not significantly different from each other, but they were significantly less than treatments AD, BD, BC, and CD. Treatment AD was significantly less than treatments BD, BC, and CD. Treatments BD and BC were not significantly different from each other, but they were significantly less than treatment CD, the treatment that generated the greatest mean EAG response (Figure 23).

For the spring emerging males, the mean EAG responses to treatments AB (1.476 mV), ABD (1.590 mV), ABCD (1.704 mV) and ABC (1.725 mV) were not significantly greater than the respective treatment's control responses (Figure 24). These treatment mixtures were not significantly different from each other, including treatment BCD (1.853 mV), which was significantly greater than its control mean. The mean EAG responses to treatment mixtures ACD (3.145 mV), AC (3.501 mV), and AD (3.966 mV) were not significantly different from each other, but they were significantly less than the mean EAG responses for treatments BC (6.116 mV), BD (6.146 mV) and CD (7.486

mV). Treatments BC and BD were not significantly different, but they were significantly less than treatment CD, the treatment with the largest mean EAG response (Figure 24).

### **Fall Weevil EAG Responses**

The same EAG procedures used on the weevils that emerged during the spring period were repeated on the weevils collected during the fall period. For the fall emerging females, the mean EAG response to the AB treatment mixture ((E)-2-hexenol + (E)-2-hexenal) was not significantly different from that of the control. However, the mean EAG responses of the remaining treatment mixtures were significantly greater than the control means (Figure 25). Regarding mixture comparisons, the mean EAG responses to treatments AB (1.672 mV), ABD ((E)-2-hexenol + (E)-2-hexenal + ethyl butyrate) (2.271 mV), ABC ((E)-2-hexenol + (E)-2-hexenal + 2-heptanone) (2.319 mV), BCD ((E)-2-hexenal + 2-heptanone + ethyl butyrate) (2.331 mV), and ABCD ((E)-2-hexenol + (E)-2-hexenal + 2-heptanone + ethyl butyrate) (2.336 mV) were not significantly different from each other, and with the exception treatment AB, they were not significantly less than the mean EAG response to treatment ACD ((E)-2-hexenol + 2-heptanone + ethyl butyrate) (3.229 mV). Treatment ACD was significantly less than treatments AC ((E)-2-hexenol + 2-heptanone) (3.391 mV) and AD ((E)-2-hexenol + ethyl butyrate) (4.300 mV). The mean EAG responses from these latter two treatments were significantly less than the mean responses to BD ((E)-2-hexenal + ethyl butyrate) (5.896 mV) and BC ((E)-2-hexenal + 2-heptanone) (6.453 mV). The treatment that had the largest mean EAG response, and which was significantly greater than all other treatments was treatment mixture CD (2-heptanone + ethyl butyrate) (7.654 mV) (Figure 25).

For the fall emerging males, the mean EAG responses to treatments AB (0.576 mV), ABD (1.377 mV), ABC (1.523 mV), BCD (1.590 mV) and ABCD (1.591 mV) were not significantly different from the control responses (Figure 26). However, the latter four treatments were not significantly different from the mean EAG responses for treatments AC (2.454 mV) and ACD (2.466 mV), which were not significant different from the AD treatment response (2.921 mV). Mean responses for treatments BD (4.297 mV) and BC (4.637 mV) were not significantly different from each other, but they were significantly less than the mean response for treatment CD (6.426 mV). Treatment CD was significantly greater than all the other treatments (Figure 26).

### **Discussion**

Chestnut odors are complex and contain a variety of volatile organic compounds that are perceived by *C. sayi*. Identification of the individual compounds within a host plant odor is critical (and at what concentrations) in order to gain some insight into how the plant-insect interaction might function (Piñero et al. 2001). Considering all sex and season groupings, the data have strongly indicated all two-compound mixtures (treatments AC, AD, BC, BD, and CD), with the exception of AB, stimulated the highest mean EAG responses; even greater than the three (treatments ABD, ABC, BCD, and ACD) and four compound mixtures (treatment ABCD). Treatment ACD did have slightly larger amplitude than treatment AC with the fall males, but the strength of two compound mixtures suggests that simply adding more compounds a weevil can detect does not lead to a larger antennal response. Weevils regardless of season of emergence and sex had particularly large responses to the mixture of 2-heptanone (C) and ethyl butyrate (D). Interestingly, among the two-compound mixtures the only one not

containing either compound, mixture AB, showed the least responsiveness. (E)-2-hexenol (A) and (E)-2-hexenal (B) when together in mixtures tended to get smaller responses suggesting a possible inhibitory effect. Mixtures of (E)-2-hexenal with 2-heptanone and ethyl butyrate consistently were the second and third largest responding mixtures further supporting interference between the 6 carbon volatiles. Additionally, female weevils responded to more of the mixtures than males for both seasonal periods with males only responding to just over half the mixtures tested. Weevil sex had different effects on different mixtures and must be considered in the development of a lure. Season of emergence had similarly divergent effects worthy of further study. The differing physiology of males and females may be responsible for differences in their antennal responses but seasonal differences are more complex. Seasonal differences may be due to further development between seasons. Examinations of *C. sayi* reproductive organs have suggested development between spring and fall emerging weevils (Keeseey unpublished data). Further investigation is required and although antennal responses do not indicate how weevils behaviorally will respond to host plant volatiles they do provide a baseline for what weevils can detect. Additionally, strength of an antennal response may be an indicator of importance and suggest behavioral bioassays for confirmation. The two-compound mixtures of (E)-2-hexenal, 2-heptanone, and ethyl butyrate consistently provided the largest most significant responses and are the best candidates for further investigation.

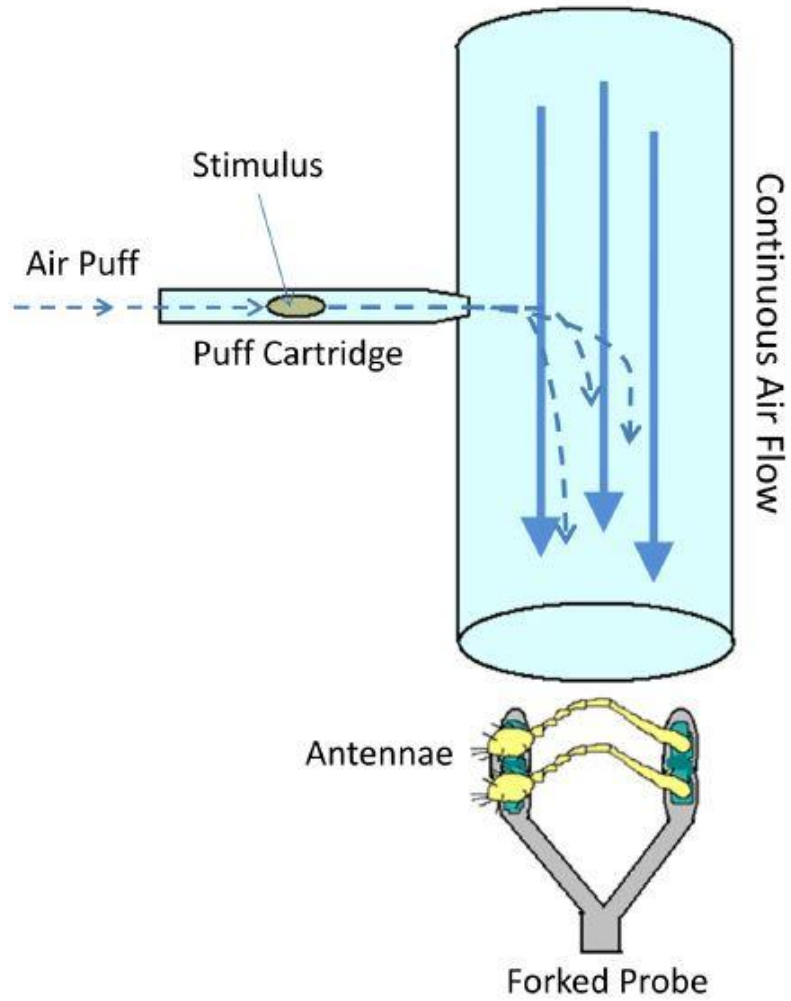
The source of the VOCs may also explain some of the differences in *C. sayi* response to mixtures. (E)-2-hexenol was produced by the catkins, while (E)-2-hexenal and 2-heptanone were both produced by the catkins and burs. Ethyl butyrate was only



produced by nut tissue. The presence of (E)-2-hexenol in the spring and ethyl butyrate in the fall however didn't seem to have a large influence on the results with a general decrease in sensitivity to all VOCs mixtures as the seasons progress. The VOCs present in bur and nut tissue (mixtures B, C, and D) in each two compound mixture did however receive the largest responses regardless of weevil sex or season of activity. The association of the nut and bur tissue with oviposition and mating could be one cause of the particularly large responses to these mixtures. Another factor that might be examined aside from the source of the VOCs is the relative ratio in produced by plant tissue. Rather than using a uniform large concentration for each VOC, attempting to replicate a ratio of compounds present may show more realistic interactions between compounds. Also more accurate ratios might negate some of the inhibitory effects present in the three-compound and four-compound mixtures.

EAG Results				
Effect	Num DF	Den DF	F value	Pr > F
<b>Sex</b>	1	83	6.55	0.0123
<b>Season</b>	1	83	6.01	0.0163
<b>Mixture</b>	10	829	111.10	<.0001
Sex*Season	1	83	1.17	0.2831
Mixture*Sex	10	829	1.27	0.2427
Mixture*Season	10	829	3.35	0.0003
Mixture*Sex*Season	10	829	1.08	0.3718

**Table 3.** Results of ANOVA performed on beetle sex, season of beetle activity, and mixtures of treatment compounds data from EAG.

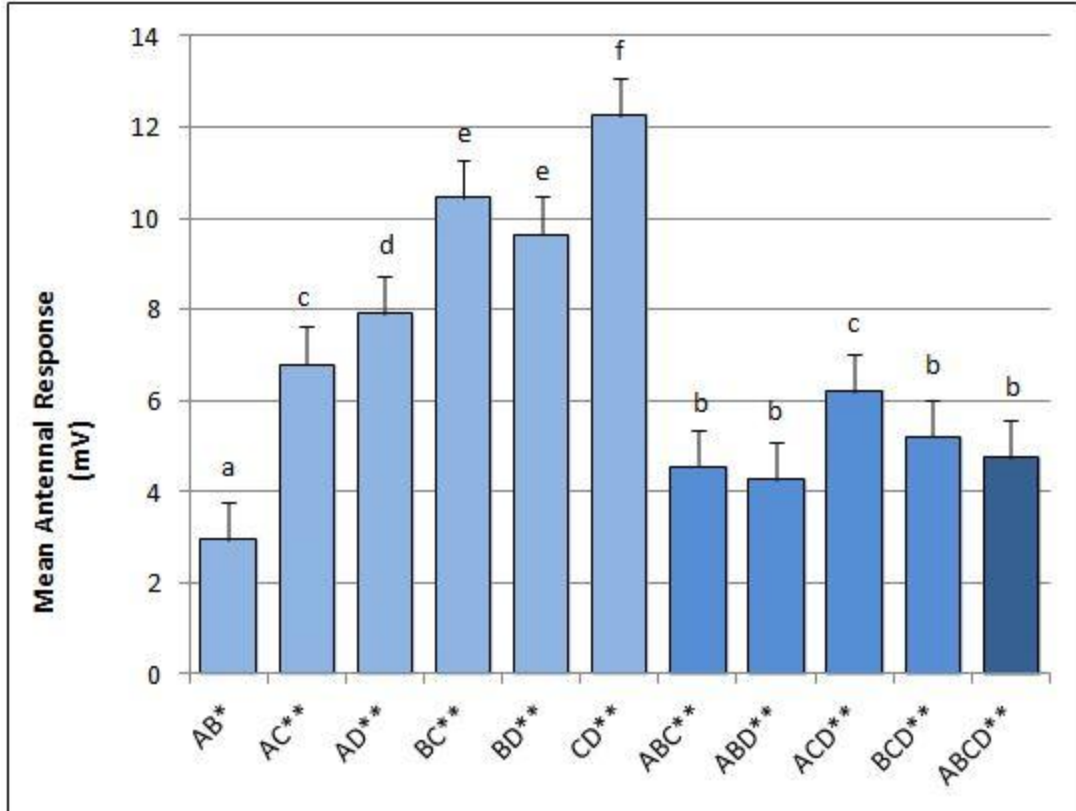


**Figure 21.** Diagram of the weevil antennal preparation (Keesey 2011).

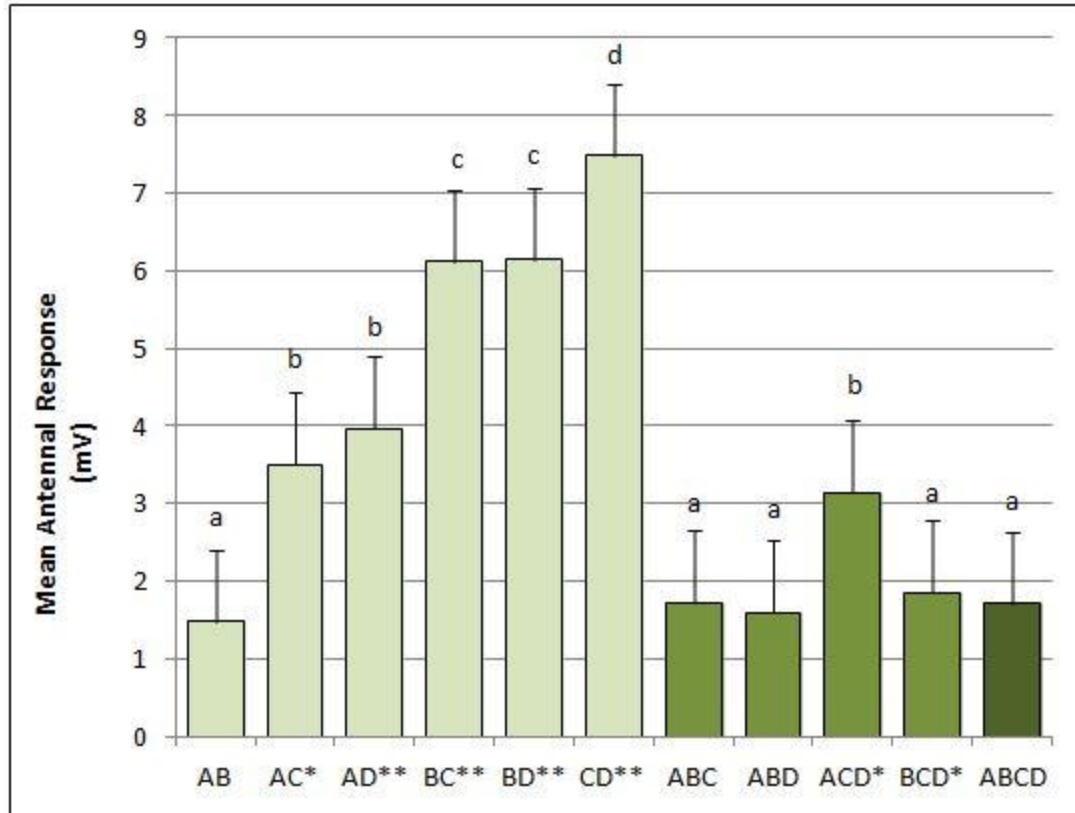


**Figure 22.** Electroantennogram (EAG) equipment. Top left: stimulus flow controller.

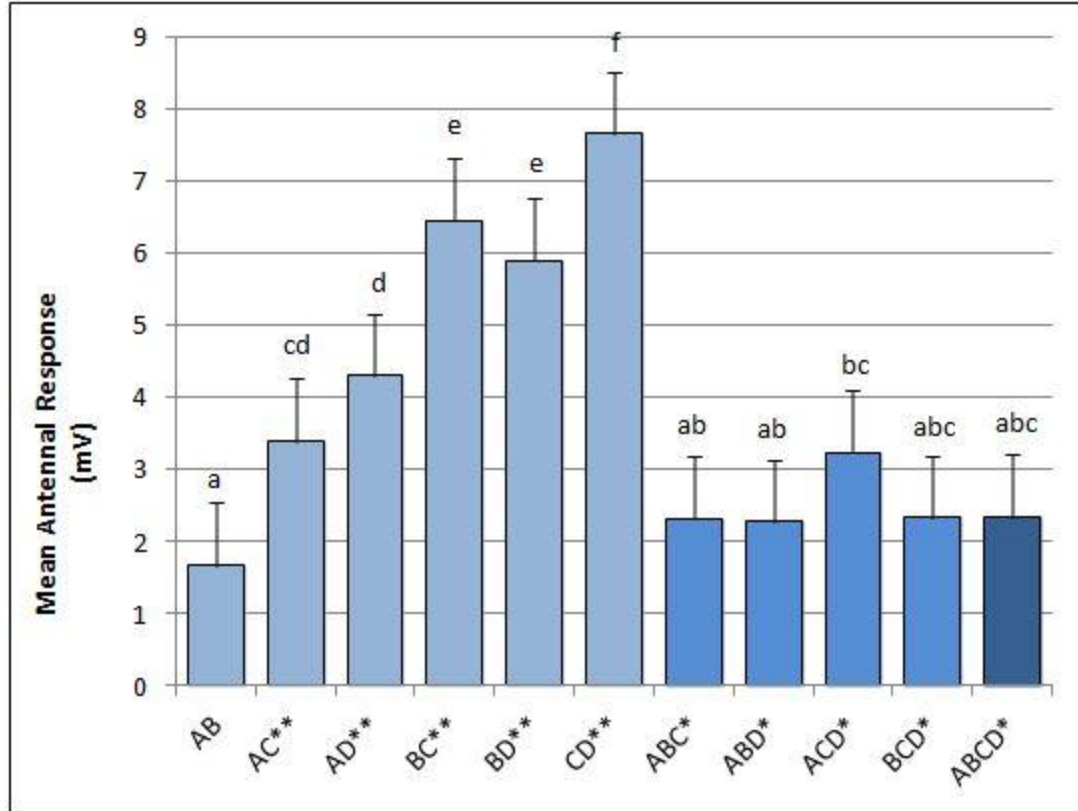
Top right: 3 dimensional micromanipulators. Bottom left: continuous airflow tube connected to GC-EAD equipment. Bottom right: IDAC-2 high-impedance amplifier and two-channel controller (images from Syntech, Hilversum, Netherlands).



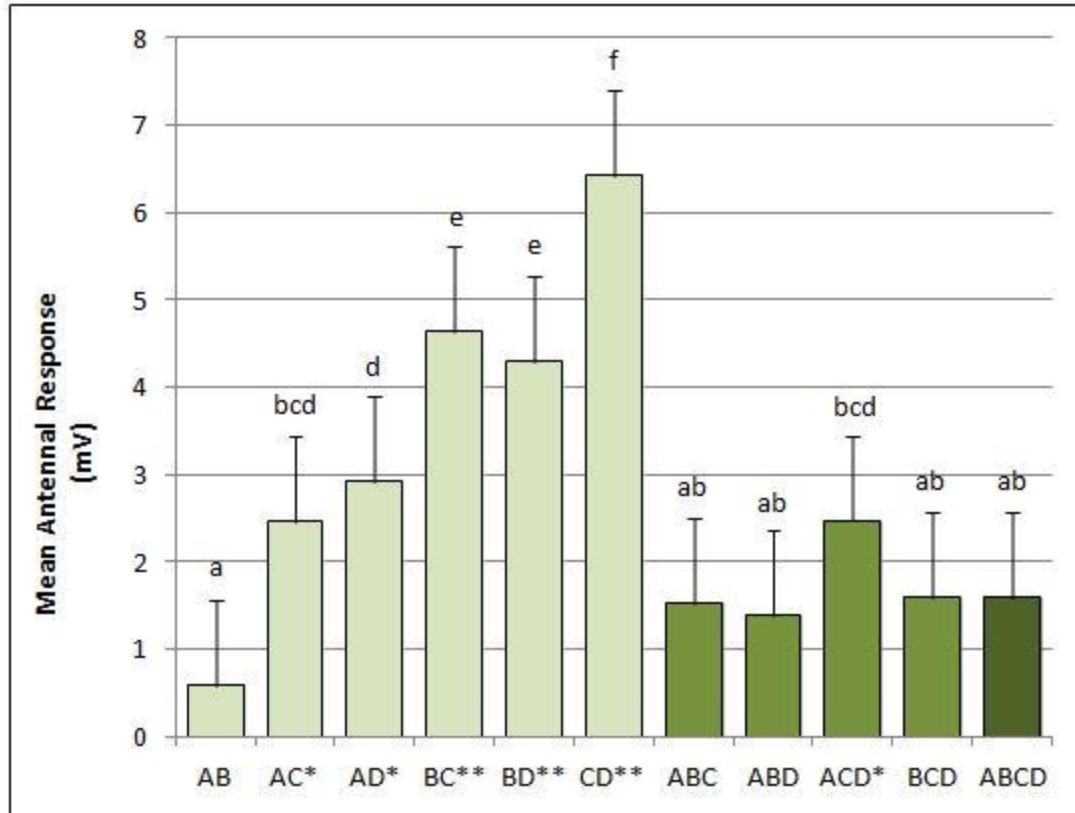
**Figure 23.** The mean ( $\pm$ SE) EAG antennal responses (mV) of female *Curculio sayi* collected during the spring emergence period (2013) to mixtures of four chestnut plant volatiles. Treatment A consists of (E)-2-hexenol, treatment B is (E)-2-hexenal, treatment C is 2-heptanone, and treatment D is ethyl butyrate. EAG amplitude means followed by the same letter are not significantly different (Fisher's protected LSD;  $P < 0.05$ ), and the presence of an asterisk(s) at each mixture description denote statistically significant differences to the corresponding controls (Fisher's protected LSD; \* $P < 0.05$ , \*\* $P < .0001$ ).



**Figure 24.** The mean ( $\pm$ SE) EAG antennal responses (mV) of male *Curculio sayi* collected during the spring emergence period (2013) to mixtures of four chestnut plant volatiles. Treatment A consists of (E)-2-hexenol, treatment B is (E)-2-hexenal, treatment C is 2-heptanone, and treatment D is ethyl butyrate. EAG amplitude means followed by the same letter are not significantly different (Fisher's protected LSD;  $P < 0.05$ ), and the presence of an asterisk(s) at each mixture description denote statistically significant differences to the corresponding controls (Fisher's protected LSD; \* $P < 0.05$ , \*\* $P < .0001$ ).



**Figure 25.** The mean ( $\pm$ SE) EAG antennal responses (mV) of female *Curculio sayi* collected during the fall emergence period (2013) to mixtures of four chestnut plant volatiles. Treatment A consists of (E)-2-hexenol, treatment B is (E)-2-hexenal, treatment C is 2-heptanone, and treatment D is ethyl butyrate. EAG amplitude means followed by the same letter are not significantly different (Fisher's protected LSD;  $P < 0.05$ ), and the presence of an asterisk(s) at each mixture description denote statistically significant differences to the corresponding controls (Fisher's protected LSD; \* $P < 0.05$ , \*\* $P < .0001$ ).



**Figure 26.** The mean ( $\pm$ SE) EAG antennal responses (mV) of male *Curculio sayi* collected during the fall emergence period (2013) to mixtures of four chestnut plant volatiles. Treatment A consists of (E)-2-hexenol, treatment B is (E)-2-hexenal, treatment C is 2-heptanone, and treatment D is ethyl butyrate. EAG amplitude means followed by the same letter are not significantly different (Fisher's protected LSD;  $P < 0.05$ ), and the presence of an asterisk(s) at each mixture description denote statistically significant differences to the corresponding controls (Fisher's protected LSD; \* $P < 0.05$ , \*\* $P < .0001$ ).



## CHAPTER V:

### EVALUATING CHESTNUT WEEVIL BEHAVIORAL RESPONSES TO SINGLE DOSE MIXTURES OF HOST PLANT VOLATILE ORGANIC COMPOUNDS (VOC) USING Y-TUBE OLFACTOMETRY

#### Introduction

The lesser (or small) chestnut weevil, *Curculio sayi* (Gyllenhal), is a key economic pest of chestnut in the central and eastern regions of the United States (Brooks and Cotton, 1929). Host plants of this native weevil species are limited to only members of the genus *Castanea* (which include chestnut and chinquapin) . But as a result of the chestnut blight caused by the fungus *Cryphonectria parasitica* in the early 20<sup>th</sup> century, the overall range of the American chestnut (*C. dentate* Marsh) was greatly reduced (Anagnostakis, 2005).

*Curculio sayi* is reported to have a 2-3 year life cycle with populations having two annual periods of activity (Brooks and Cotton, 1929; Johnson, 1956; Keesey and Barrett, 2008). The first activity period occurs in the spring when the chestnut catkins are blooming. During this time the adult weevils emerge from the ground and move into the trees and feed on the catkins. Adult emergence from the soil decreases about the time the catkins enter senescence. During this time the adult weevils leave the tree and, presumably, return to the ground debris where they enter a period of inactivity (Anagnostakis, 2005).

During late-summer/early-fall when the chestnut burs begin to fully form and split, the adult weevils that had been resting in the duff on the ground (and other secluded sites) become active again and return to the chestnut tree canopy. Additionally, Keesey and Barrett (2008) reported that during this period in the fall a second (but smaller)

emergence of adults from the soil also occurs. After mating the female weevil begins to lay eggs (usually in September) by chewing a hole through the nut and sometimes the bur. The developing larvae will feed on the nut contents for about 3 weeks, after which it will emerge from the nut and burrow into the soil to pupate (Brooks and Cotton 1929, Johnson 1956).

Rodriguez-Saona and Stelinski (2009) suggested that before host-plant volatile organic compounds (VOCs) can be effectively utilized in integrated pest management (IPM) programs, such as a monitoring trap attractant, an understanding of the behavioral responses of the target pest towards the VOCs must be known. Previous research (Keesey 2013, Keesey and Barrett 2012) identified eight chestnut volatile organic compounds (VOCs) as being potentially attractive to *C. sayi*. The purpose of this study was to evaluate the level of behavioral activity of *C. sayi* towards mixtures of some of these key host plant VOCs using Y-tube olfactometry, at a single dose, and to compare the behavioral responses across weevil sex and season of adult activity.

## **Materials and Methods**

### **Field Site and Weevil Collection**

Adult *Curculio sayi* were collected from a private farm near the city of Glasgow within Saline County (39.19190° N, 292.93110° W), Missouri. Planted on the farm were several different nut trees including several different chestnuts (*Castanea spp.*). The USDA soil type at the farm was Menfro silt loam. The site contains 14 chestnuts of different varieties spaced between 7 to 10 meters apart with overlapping canopies. The trees are 15-18 m tall and were estimated between 40 and 50 years of age and a grafted variety cross of Asian and American chestnut species (Ken Hunt, correspondence). The

tree's catkins grow through April to June and nuts begin to drop in August continuing all the way through October.

*C. sayi* were collected using three types of traps: ground-based emergence traps, tree-mounted circle traps and silhouette traps (for detailed descriptions of trap specifics see Keesey 2008). Additionally, a limb-tapping technique with canvas drop cloths to catch falling weevils dislodged from the canopy was employed. Once collected weevils were sexed using proboscis length and shape (a sexually dimorphic trait) and then separated for transport back to the laboratory. In the laboratory the weevils were stored in plastic half liter cups containing 2-3 cm bedding of pine wood shavings. Two sponge cubes saturated with honey water were kept in each cup, which contained about 12 weevils (same sex). Cups were stored in a growth chamber set with a 14:10 (L:D) hour photoperiod and a temperature of 27° C. Collected weevils were tested only during the same period as their emergence, i.e. no spring weevils were tested or utilized during the fall testing period.

### **Y-tube Olfactometry**

The Y-tube olfactometer consisted of glassware of two 10 cm arms connected to a 15 cm stem (24 mm diameter) (Analytical Research Systems, Gainesville, FL) (Figure 1). Compressed air was humidified and filtered with active charcoal then passed through two inline flow meters. The flow meters controlled the airflow through either arm of the Y-tube at a rate of 0.5 liter/min. Glass holding chambers (15 cm in length by 3 cm in diameter) were used to introduce 1  $\mu$ L treatment and control odors on filter paper wedges (Whatman No.4) into the air flow with connections made with Teflon tubing. The assembly was centered about 3 m beneath a fluorescent light fixture containing two 1 m

long 32-watt bulbs producing between 310 and 340 lux. The Y-tube was held at a 30° angle in a white-walled cardboard enclosure to prevent the interference of visual cues.

Individual weevils were introduced to the Y-tube using a glass release chamber connected to the Y-tube stem. A choice was recorded when an insect traveled up the stem and into the end of either of the arms of the Y-tube. If within 5 minutes the insect did not reach the end of one of the Y-tube arms, the weevil was removed and a 'no choice' was recorded. There were at least 10 replications per treatment with weevils being tested twice at both positions of the odor source (the Y-tube was flipped to prevent any directional bias). Tested *C. sayi* were fasted 24 hours prior and given a recovery period of at least 24 hours after testing. Due to the limited number of weevils, previously tested weevils were reused for further repetitions (the 48 hour periods between tests acted to negate any past interference). All Y-tube glassware was cleaned with hot soapy water and rinsed with methanol and acetone before being left to air-dry overnight.

### **Solution Preparation and Data Analysis**

The chestnut VOCs that were evaluated were (E)-2-hexenol (hereafter referred to as treatment A), (E)-2-hexenal (hereafter referred to as treatment B), 2-heptanone (hereafter referred to as treatment C), and ethyl butyrate (hereafter referred to as treatment D). Treatment solutions were prepared at a 1:10 dilution, compound to solvent, based on the responsiveness of individual volatiles to the solvent laboratory grade mineral oil (Sigma Aldrich, St. Louis, MO). The purity of each of the synthetic VOCs was high (over 95%). In addition, mixtures consisting of two, three and four of the compounds were also prepared. Fresh treatment solutions were prepared each day for weevils to be tested.

The data were analyzed using a logistic analysis of variance (ANOVA) to compare treatment means (PROC GENMOD; SAS v. 9.3, SAS Institute Inc., Cary, NC). Treatments were arranged as an 11 x 2 x 2 factorial with compound mixture by sex of weevil by season of weevil activity. The mean differences in the 3-way factorial were determined using least-squares means (PROC GENMOD; LSMEANS; SAS v. 9.2).

## **Results**

Y-tube bioassay responses from female and male weevils collected during the spring and fall emergence periods (2013) were analyzed individually by sex with comparisons of the compound mixtures. Overall, there was a high level of responsiveness with only 20% of weevils not making a choice considering all of the trials.

The analysis of variance showed significant effects ( $P < 0.05$ ) individually for mixture, sex and season (Table 4). Among the possible two-way interactions, only the interaction of sex and season was significant. Also, the three-way interaction of all explanatory variables was not significant.

### **Two-component mixtures**

For the AB mixture ((E)-2-hexenol + (E)-2-hexenal), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 8 replicates chose the control and 10 chose the treatment; and for the males, 9 replicates chose the control arm and 9 chose the treatment. For the fall emerging weevils, 13 of the female replicates chose the control and 16 selected the treatment. The males had 11 replicates choosing the control and 9 selecting the treatment (Figure 28). There

were no significant differences between the control and treatment choices regardless of weevil sex and period of activity.

Considering only the seasonal period of activity (weevil sex combined) in response to the AB mixture, spring emerging weevils chose the control arm 17 times and the treatment arm 19 times (Figure 29); and the fall emerging weevils chose the control 24 times and the treatment 25 times (Figure 30). There were no significant differences between the control and treatment responses for both the spring and fall emerging weevils ( $P < 0.05$ ).

When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the AB mixture, female weevils chose the control arm 21 times and the treatment arm 26 times (Figure 31); and the male weevils chose the control 20 times and the treatment 18 times (Figure 32). There were no significant differences between the control and treatment responses for both the female and male weevils ( $P < 0.05$ ).

For the AC mixture ((E)-2-hexenol + 2-heptanone), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 11 replicates chose the control and 11 chose the treatment; and for the males, 8 replicates chose the control arm and 12 chose the treatment. For the fall emerging weevils, 13 of the female replicates chose the control and 7 selected the treatment. The males had 13 replicates choosing the control and 8 selecting the treatment (Figure 33). There were no significant differences between the control and treatment choices regardless of weevil sex and period of activity.

Considering only the seasonal period of activity (weevil sex combined) in response to the AC mixture, spring emerging weevils chose the control arm 19 times and the treatment arm 23 times (Figure 29); and the fall emerging weevils chose the control 26 times and the treatment 15 times (Figure 30). There were no significant differences between the control and treatment responses for both the spring and fall emerging weevils ( $P < 0.05$ ).

When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the AC mixture, female weevils chose the control arm 24 times and the treatment arm 18 times (Figure 31); and the male weevils chose the control 21 times and the treatment 20 times (Figure 32). There were no significant differences between the control and treatment responses for both the female and male weevils ( $P < 0.05$ ).

For the AD mixture ((E)-2-hexenol + ethyl butyrate), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 10 replicates chose the control and 5 chose the treatment; and for the males, 8 replicates chose the control arm and 12 chose the treatment. For the fall emerging weevils, 9 of the female replicates chose the control and 15 selected the treatment. The males had 5 replicates choosing the control and 9 selecting the treatment (Figure 34). There were no significant differences between the control and treatment choices regardless of weevil sex and period of activity.

Considering only the seasonal period of activity (weevil sex combined) in response to the AD mixture, spring emerging weevils chose the control arm 18 times and the treatment arm 17 times (Figure 29); and the fall emerging weevils chose the control 14

times and the treatment 14 times (Figure 30). There were no significant differences between the control and treatment responses for both the spring and fall emerging weevils ( $P < 0.05$ ).

When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the AD mixture, female weevils chose the control arm 19 times and the treatment arm 10 times (Figure 31); and the male weevils chose the control 13 times and the treatment 21 times (Figure 32). There were no significant differences between the control and treatment responses for both the female and male weevils ( $P < 0.05$ ).

For the BC mixture ((E)-2-hexenal + 2-heptanone), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 6 replicates chose the control and 13 chose the treatment; and for the males, 8 replicates chose the control arm and 18 chose the treatment. For the fall emerging weevils, 13 of the female replicates chose the control and 7 selected the treatment. The males had 6 replicates choosing the control and 6 selecting the treatment (Figure 35). There were no significant differences between the control and treatment choices regardless of weevil sex and period of activity.

Considering only the seasonal period of activity (weevil sex combined) in response to the BC mixture, spring emerging weevils chose the control arm 14 times and the treatment arm 31 times (Figure 29); and the fall emerging weevils chose the control 19 times and the treatment 13 times (Figure 30). There were significant differences between the control and treatment responses for the spring active weevils ( $P < 0.05$ ).



When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the BC mixture, female weevils chose the control arm 19 times and the treatment arm 20 times (Figure 31); and the male weevils chose the control 14 times and the treatment 24 times (Figure 32). There were no significant differences between the control and treatment responses for both the female and male weevils ( $P < 0.05$ ).

For the BD mixture ((E)-2-hexenal + ethyl butyrate), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 8 replicates chose the control and 11 chose the treatment; and for the males, 5 replicates chose the control arm and 8 chose the treatment. For the fall emerging weevils, 13 of the female replicates chose the control and 10 selected the treatment. The males had 11 replicates choosing the control and 10 selecting the treatment (Figure 36). There were no significant differences between the control and treatment choices regardless of weevil sex and period of activity.

Considering only the seasonal period of activity (weevil sex combined) in response to the BD mixture, spring emerging weevils chose the control arm 13 times and the treatment arm 19 times (Figure 29); and the fall emerging weevils chose the control 19 times and the treatment 13 times (Figure 30). There were no significant differences between the control and treatment responses for both the spring and fall emerging weevils ( $P < 0.05$ ).

When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the BD mixture, female weevils chose the control arm 21 times and the treatment arm 21 times (Figure 31); and the male weevils chose the

control 16 times and the treatment 18 times (Figure 32). There were no significant differences between the control and treatment responses for both the female and male weevils ( $P < 0.05$ ).

For the CD mixture (2-heptanone + ethyl butyrate), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 10 replicates chose the control and 7 chose the treatment; and for the males, 13 replicates chose the control arm and 9 chose the treatment. For the fall emerging weevils, 15 of the female replicates chose the control and 7 selected the treatment. The males had 7 replicates choosing the control and 11 selecting the treatment (Figure 37). There were no significant differences between the control and treatment choices regardless of weevil sex and period of activity.

Considering only the seasonal period of activity (weevil sex combined) in response to the CD mixture, spring emerging weevils chose the control arm 23 times and the treatment arm 16 times (Figure 29); and the fall emerging weevils chose the control 22 times and the treatment 18 times (Figure 30). There were no significant differences between the control and treatment responses for both the spring and fall emerging weevils ( $P < 0.05$ ).

When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the CD mixture, female weevils chose the control arm 25 times and the treatment arm 14 times (Figure 31); and the male weevils chose the control 20 times and the treatment 20 times (Figure 32). There were no significant differences between the control and treatment responses for both the female and male weevils ( $P < 0.05$ ).

### **Three-component mixtures**

For the ABC mixture ((E)-2-hexenol + (E)-2-hexenal + 2-heptanone), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 8 replicates chose the control and 3 chose the treatment; and for the males, 7 replicates chose the control arm and 7 chose the treatment. For the fall emerging weevils, 16 of the female replicates chose the control and 9 selected the treatment. The males had 12 replicates choosing the control and 9 selecting the treatment (Figure 38). There were no significant differences between the control and treatment choices regardless of weevil sex and period of activity.

Considering only the seasonal period of activity (weevil sex combined) in response to the ABC mixture, spring emerging weevils chose the control arm 15 times and the treatment arm 10 times (Figure 29); and the fall emerging weevils chose the control 28 times and the treatment 18 times (Figure 30). There were no significant differences between the control and treatment responses for both the spring and fall emerging weevils ( $P < 0.05$ ).

When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the ABC mixture, female weevils chose the control arm 24 times and the treatment arm 12 times (Figure 31); and the male weevils chose the control 19 times and the treatment 16 times (Figure 32). There were significant differences between the control and treatment responses for only the females ( $P < 0.05$ ).

For the ABD mixture ((E)-2-hexenol + (E)-2-hexenal + ethyl butyrate), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 11 replicates chose the control and 5 chose the

treatment; and for the males, 15 replicates chose the control arm and 6 chose the treatment. For the fall emerging weevils, 5 of the female replicates chose the control and 8 selected the treatment. The males had 9 replicates choosing the control and 12 selecting the treatment (Figure 39). There were no significant differences between the control and treatment choices regardless of weevil sex and period of activity.

Considering only the seasonal period of activity (weevil sex combined) in response to the ABD mixture, spring emerging weevils chose the control arm 26 times and the treatment arm 11 times (Figure 29); and the fall emerging weevils chose the control 14 times and the treatment 20 times (Figure 30). There were significant differences between the control and treatment responses for the spring active weevils ( $P < 0.05$ ).

When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the ABD mixture, female weevils chose the control arm 16 times and the treatment arm 13 times (Figure 31); and the male weevils chose the control 24 times and the treatment 18 times (Figure 32). There were no significant differences between the control and treatment responses for both the female and male weevils ( $P < 0.05$ ).

For the ACD mixture ((E)-2-hexenol + 2-heptanone + ethyl butyrate), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 4 replicates chose the control and 1 chose the treatment; and for the males, 11 replicates chose the control arm and 3 chose the treatment. For the fall emerging weevils, 12 of the female replicates chose the control and 8 selected the treatment. The males had 12 replicates choosing the control and 12 selecting the

treatment (Figure 40). There were no significant differences between the control and treatment choices regardless of weevil sex and period of activity, with the exception of the spring emerging males ( $P < 0.05$ ).

Considering only the seasonal period of activity (weevil sex combined) in response to the ACD mixture, spring emerging weevils chose the control arm 15 times and the treatment arm 4 times (Figure 29); and the fall emerging weevils chose the control 24 times and the treatment 20 times (Figure 30). There were significant differences between the control and treatment responses for the spring active weevils ( $P < 0.05$ ).

When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the ACD mixture, female weevils chose the control arm 16 times and the treatment arm 9 times (Figure 31); and the male weevils chose the control 23 times and the treatment 15 times (Figure 32). There were no significant differences between the control and treatment responses for both the female and male weevils ( $P < 0.05$ ).

For the BCD mixture ((E)-2-hexenal + 2-heptanone + ethyl butyrate), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 10 replicates chose the control and 6 chose the treatment; and for the males, 9 replicates chose the control arm and 13 chose the treatment. For the fall emerging weevils, 8 of the female replicates chose the control and 13 selected the treatment. The males had 10 replicates choosing the control and 16 selecting the treatment (Figure 41). There were no significant differences between the control and treatment choices regardless of weevil sex and period of activity.

Considering only the seasonal period of activity (weevil sex combined) in response to the BCD mixture, spring emerging weevils chose the control arm 19 times and the treatment arm 19 times (Figure 29); and the fall emerging weevils chose the control 18 times and the treatment 29 times (Figure 30). There were no significant differences between the control and treatment responses for both the spring and fall emerging weevils ( $P < 0.05$ ).

When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the BCD mixture, female weevils chose the control arm 18 times and the treatment arm 19 times (Figure 31); and the male weevils chose the control 19 times and the treatment 29 times (Figure 32). There were no significant differences between the control and treatment responses for both the female and male weevils ( $P < 0.05$ ).

#### **Four-component mixture**

For the ABCD mixture ((E)-2-hexenol + (E)-2-hexenal + 2-heptanone + ethyl butyrate), the response of the spring emerging weevils (either choosing the control or the treatment) was as follows. For the females, 14 replicates chose the control and 2 chose the treatment; and for the males, 17 replicates chose the control arm and 5 chose the treatment. For the fall emerging weevils, 11 of the female replicates chose the control and 5 selected the treatment. The males had 10 replicates choosing the control and 7 selecting the treatment (Figure 42). There were no significant differences between the control and treatment choices regardless of weevil sex and period of activity, with the exception of the spring emerging females and males ( $P < 0.05$ ).

Considering only the seasonal period of activity (weevil sex combined) in response to the ABCD mixture, spring emerging weevils chose the control arm 31 times and the treatment arm 7 times (Figure 29); and the fall emerging weevils chose the control 21 times and the treatment 12 times (Figure 30). There were significant differences between the control and treatment responses for only the spring active weevils ( $P < 0.05$ ).

When considering only the sex of the weevil (seasonal period of activity combined) regarding the response to the ABCD mixture, female weevils chose the control arm 25 times and the treatment arm 7 times (Figure 31); and the male weevils chose the control 27 times and the treatment 12 times (Figure 32). There were significant differences between the control and treatment responses for both the female and male weevils ( $P < 0.05$ ).

### **Discussion**

The Y-tube bioassays using mixtures of compounds provided several significant responses. The mixture of all the selected volatiles, ABCD ((E)-2-hexenol + (E)-2-hexenal + 2-heptanone + ethyl butyrate), showed significant responses but only in the spring. The only other significant response when considering both sex and season of the weevil was ACD from spring males. It is interesting both that the mixture with the most VOCs (ABCD) and most different VOC combination (ACD) receive significant responses. Host plant odors are often made up of components with synergistic effects (Visser 1986, Hansson 2002) suggesting that the responses may have been due to interactions between multiple compounds. (E)-2-hexenol and (E)-2-hexenal are more similar than the other two VOCs and appeared to interfere with each other at least from

the antennal responses (see chapter IV). The behavioral results however, did not appear to follow a similar pattern with the most responsive mixtures being those that contained the most compounds. More control than treatment choices suggest that the mixtures are repellent rather than attractive, however. Also, the significant responses were from spring *C. sayi* suggesting that spring-active weevils may be more responsive to host plant odors. Despite this the overall unresponsiveness may be an issue with nearly all treatments not responding significantly regardless of sex or season. Due to the limited amount of weevils from a single site more behavioral bioassay repetitions could not be performed but possibly have provided clearer results. Disregarding both sex and season increased the experimental power and showed similar significance when considering those variables. Spring weevils were responsive while fall weevils were not and male weevils had slightly more significant responses than females. All these significant responses, however, should be investigated further to confirm there was no interference from the relatively low power. The combination of relatively low repetitions and large number of treatments suggests that at least some of the treatments were likely to be significant if responses were random. Despite this possibility, measures were taken to control outside interference and EAG results suggest non-random behavioral responses. The responses recorded suggest that EAG responses, while they may indicate a weevil's ability to detect a compound, may be insufficient to determine a VOCs effect or more specifically interactions between compounds behaviorally. The behavioral results suggest that further study should focus on a combination of multiple VOCs, and improper ratios or volatile concentrations may be repellent to weevils. The mixture, season of activity, and sex were all significant effects independently in weevil behavioral



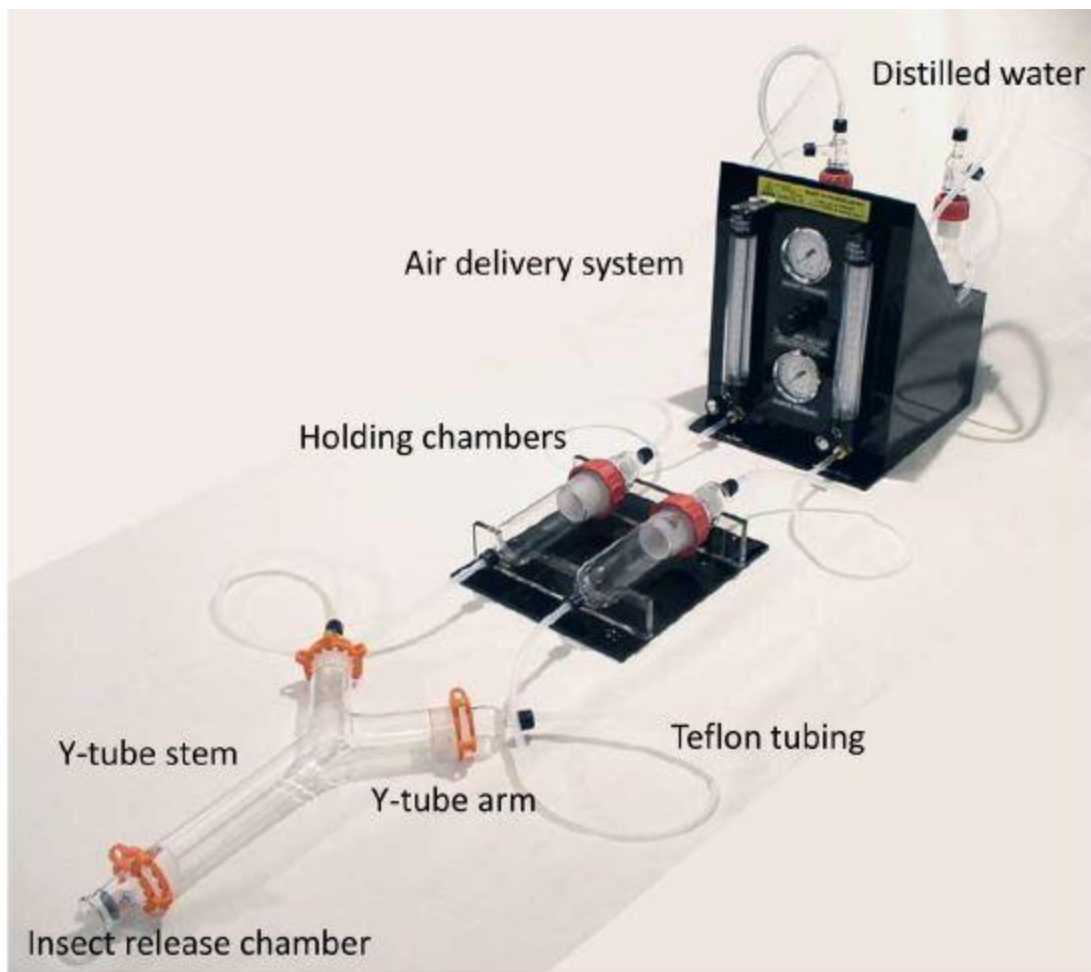
responses. Additionally, the interaction between sex and season suggest that weevils react differently depending on their sex as the season progresses. As mentioned previously (Chapter IV), dissection and measurements taken of *C. sayi* reproductive organs differed between seasonal periods of adult activity (Keesey, unpublished data). These physiological changes and the maturation of weevils may be responsible for changes in weevil behavior.

Further behavioral testing may have more success with the use of different ratios of VOCs in mixtures rather than maintaining a uniform concentration. The uniform concentration was selected largely because physiologically weevils responded to each VOC strongly but aligning the mixtures more closely with what weevils might experience naturally could lead to attraction. One of the challenges of using different concentrations ratios is that different plant tissues produced the selected VOCs. (E)-2-hexenal for instance, was only produced by the catkins and wouldn't be present at the same as ethyl butyrate, which was only produced by nut tissue. This may be another explanation for the repellent responses of *C. sayi* to the mixture ABCD, which included both compounds. Considering both of these factors the best solution may be to either stick to compounds produced by multiple plant tissues (i.e. (E)-2-hexenal and 2-heptanone) or attempt to replicate the important VOCs from a single plant tissue.

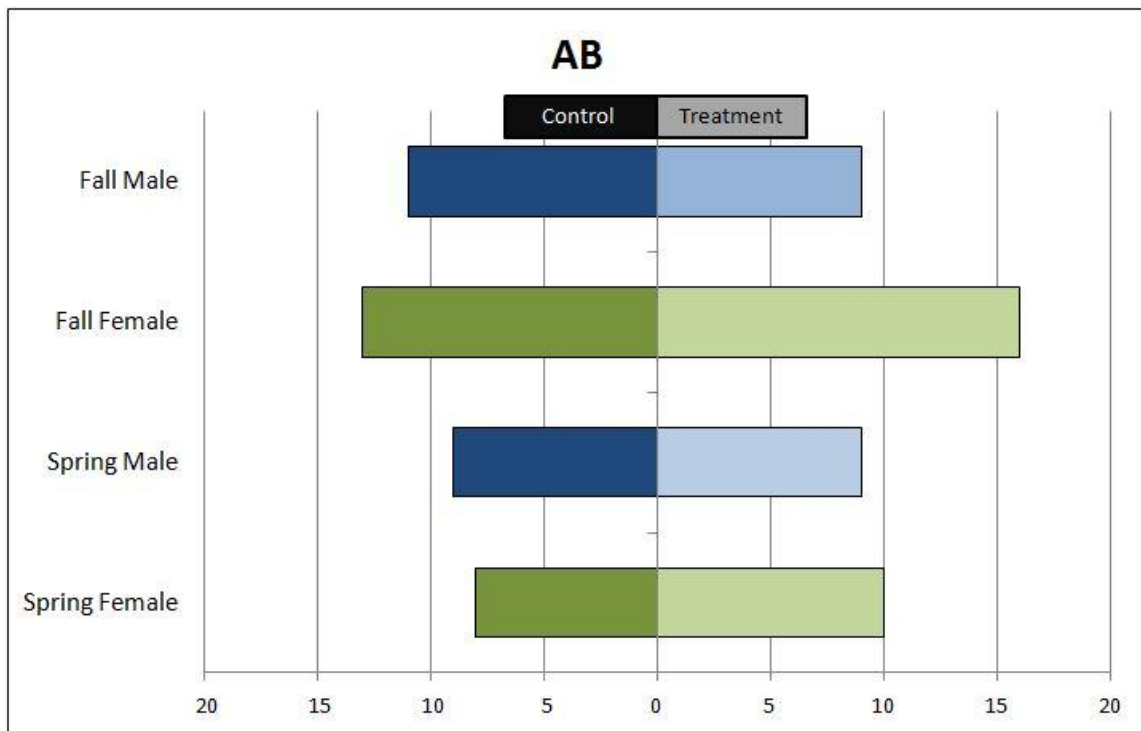
### Y-tube Bioassay Results

Effect	Num DF	Chi-square	Pr > Chi Sq
<b>Mixture</b>	10	24.20	0.0123
<b>Season</b>	1	1.11	0.0163
<b>Sex</b>	1	5.25	<.0001
Mixture*Season	10	23.13	0.2831
Treatment*Sex	10	6.47	0.2427
Season*Sex	1	0.00	0.0003
Treatment*Season*Sex	10	3.75	0.3718

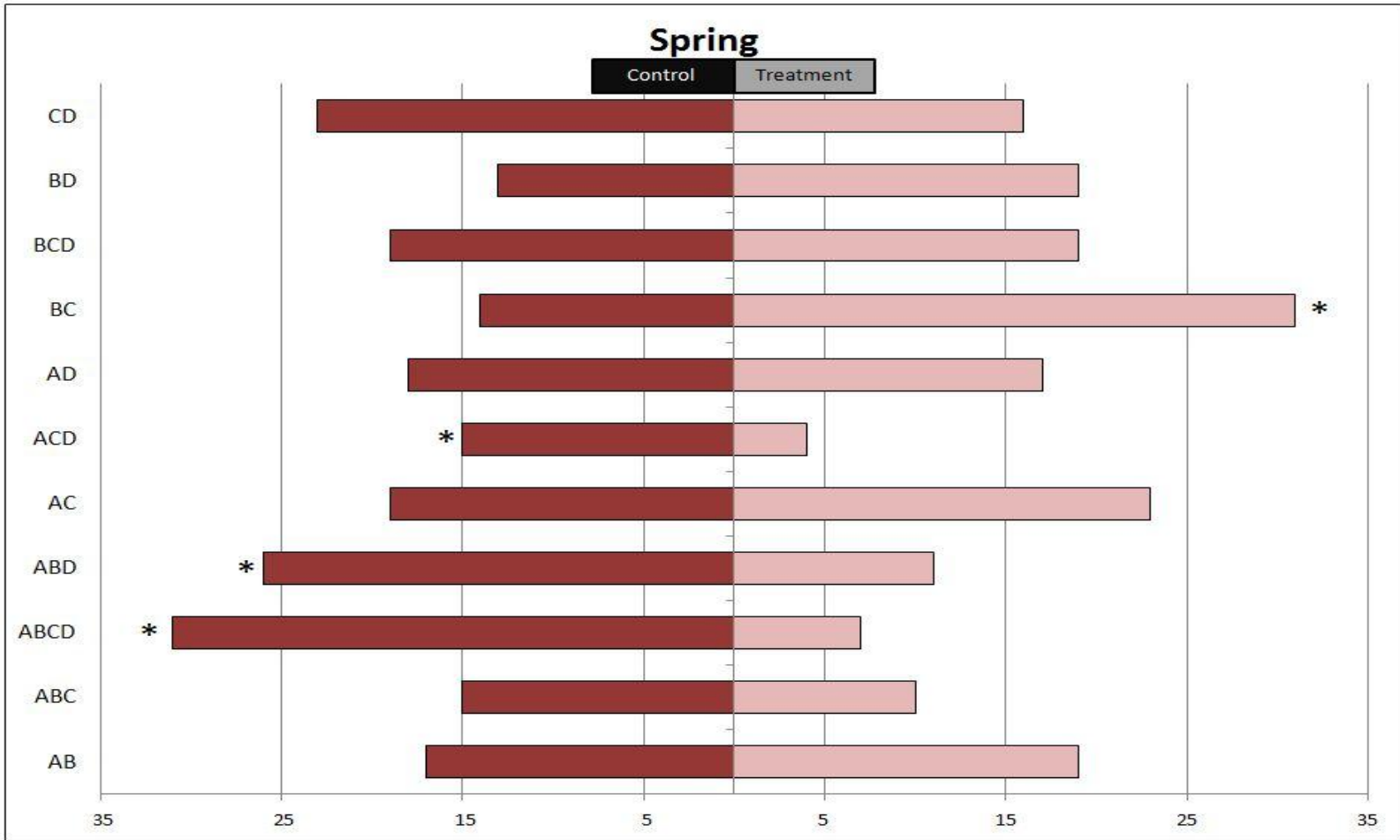
**Table 4.** Results of ANOVA performed on mixtures of treatment compounds, season of beetle activity, and beetle sex on data from Y-tube bioassays.



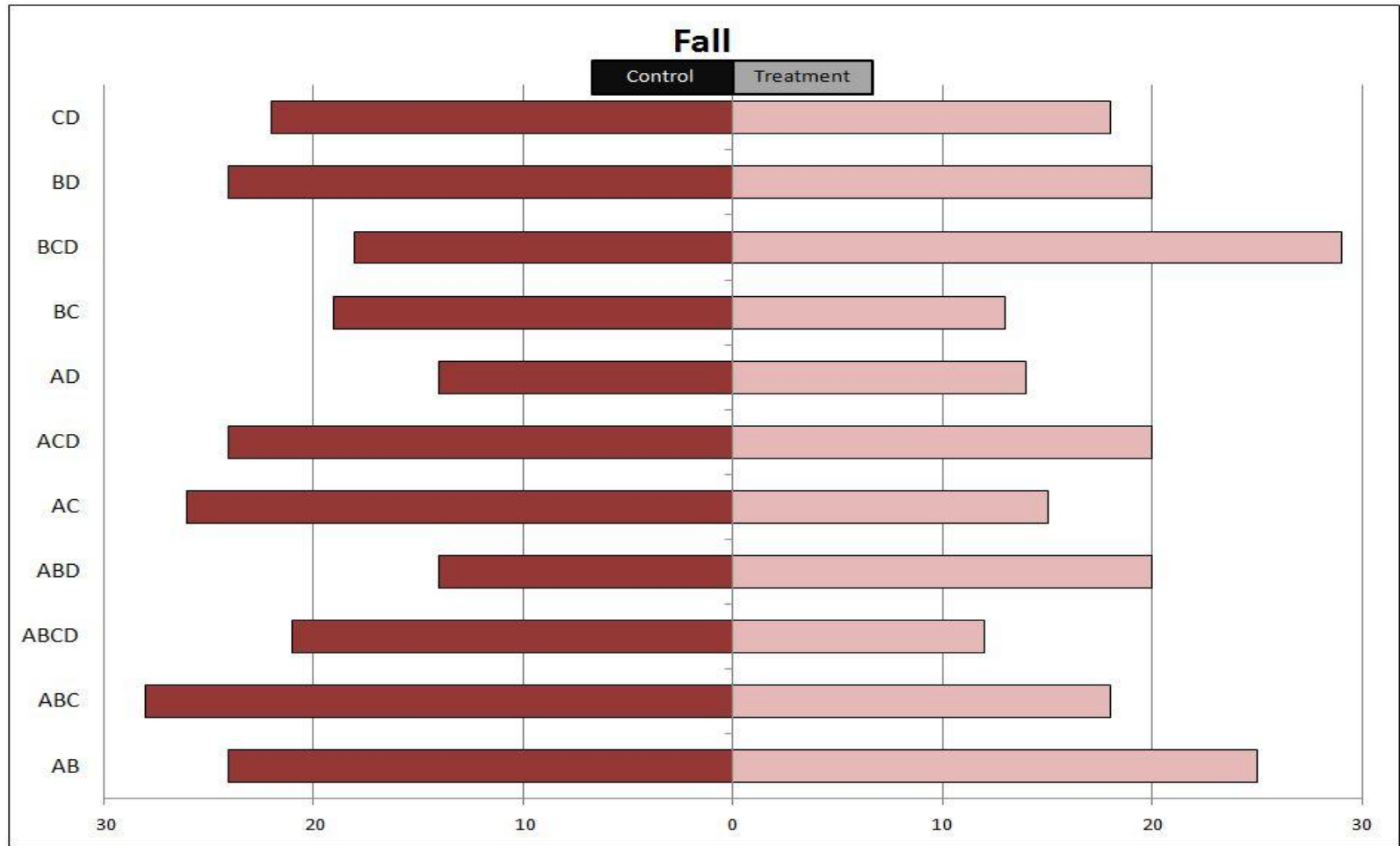
**Figure 27.** Y-tube olfactometer including air filtering and flow controllers along with treatment release chamber (image from Analytical Research Systems, Inc., Gainesville, FL).



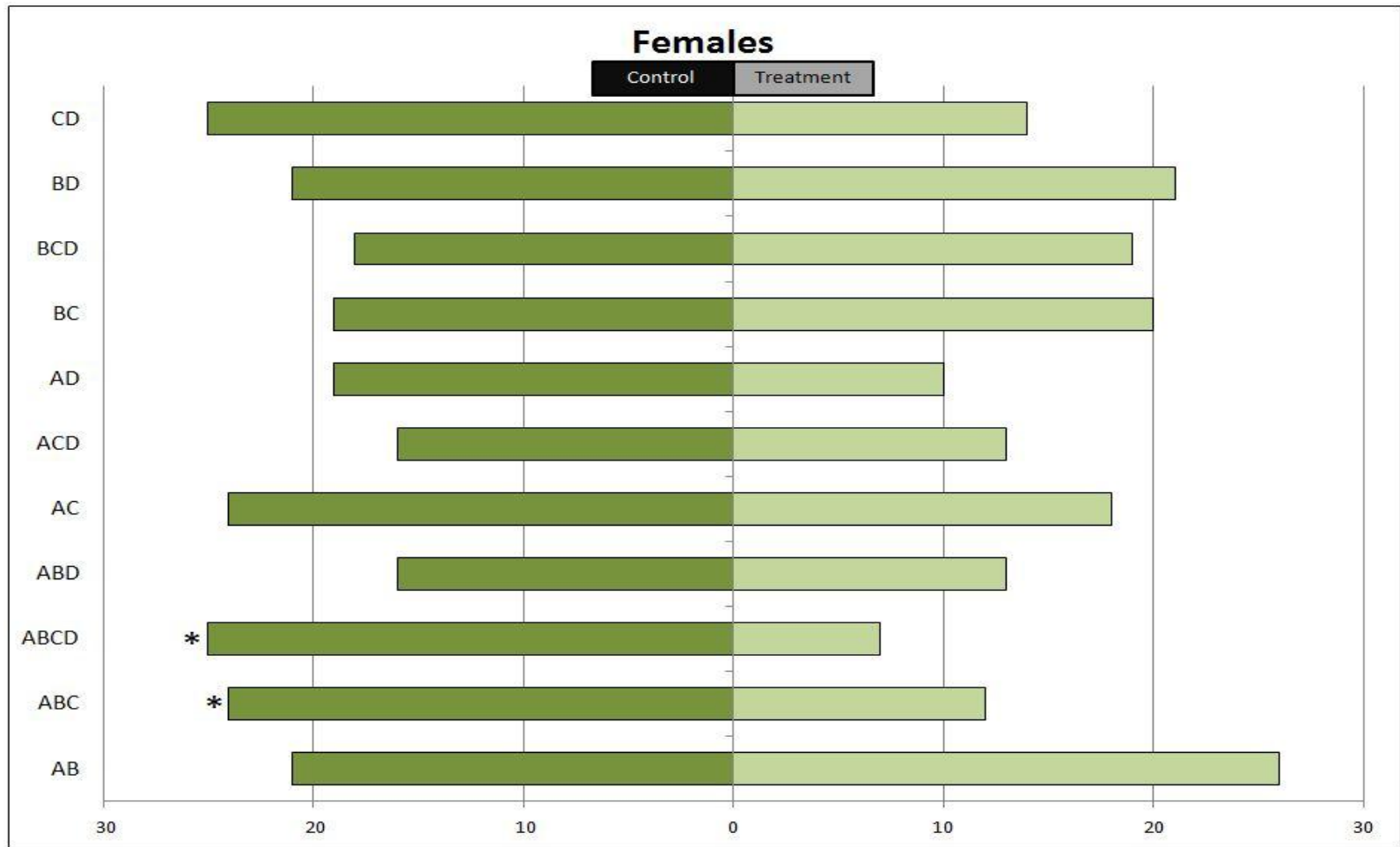
**Figure 28.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of adult activity towards the mixture of (E)-2-hexenol and (E)-2-hexenal. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between control and treatment responses.



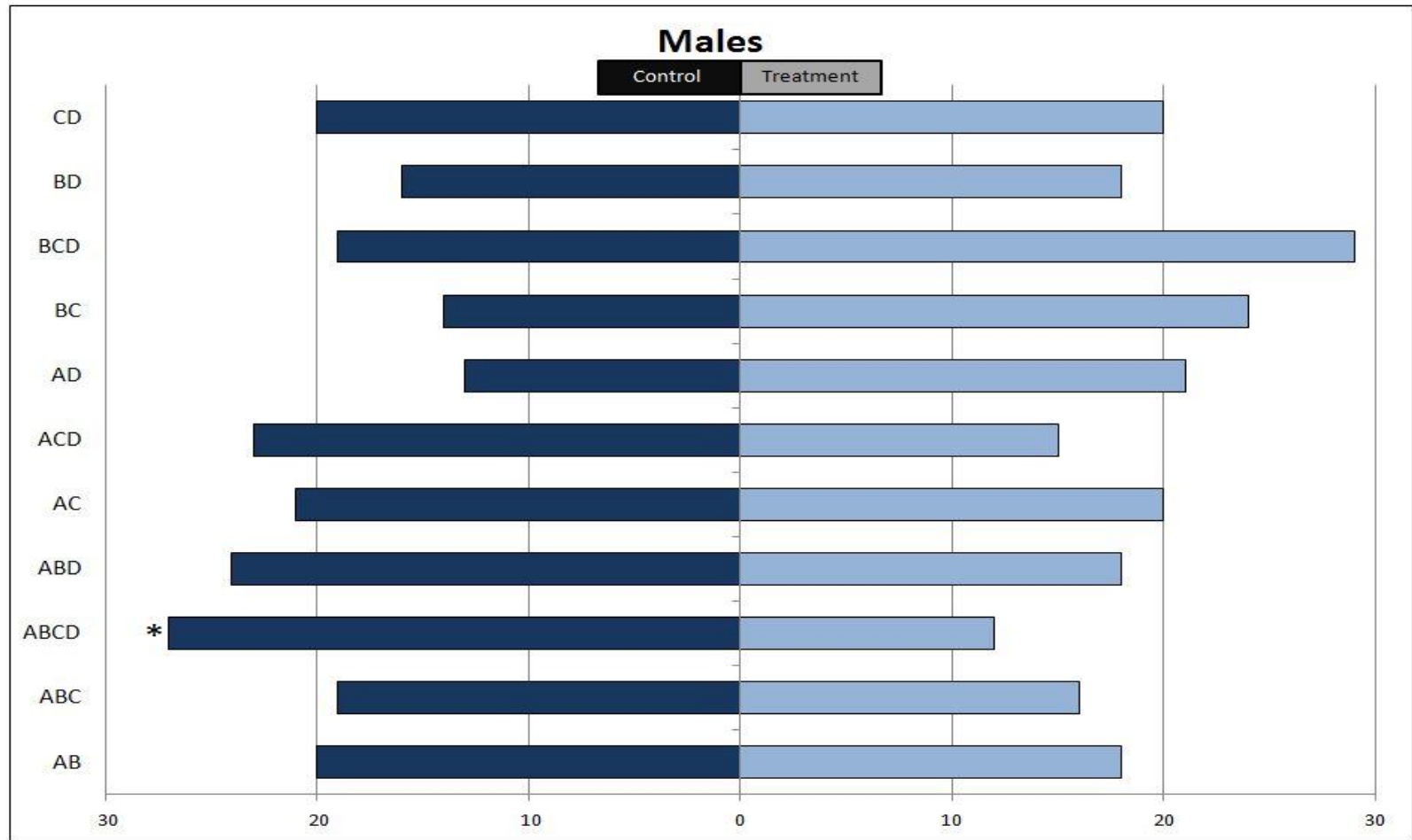
**Figure 29.** Y-tube olfactometer responses of spring-active *C. sayi* towards each mixture of compounds regardless of sex. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



**Figure 30.** Y-tube olfactometer responses of fall-active *C. sayi* towards each mixture of compounds regardless of sex. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.

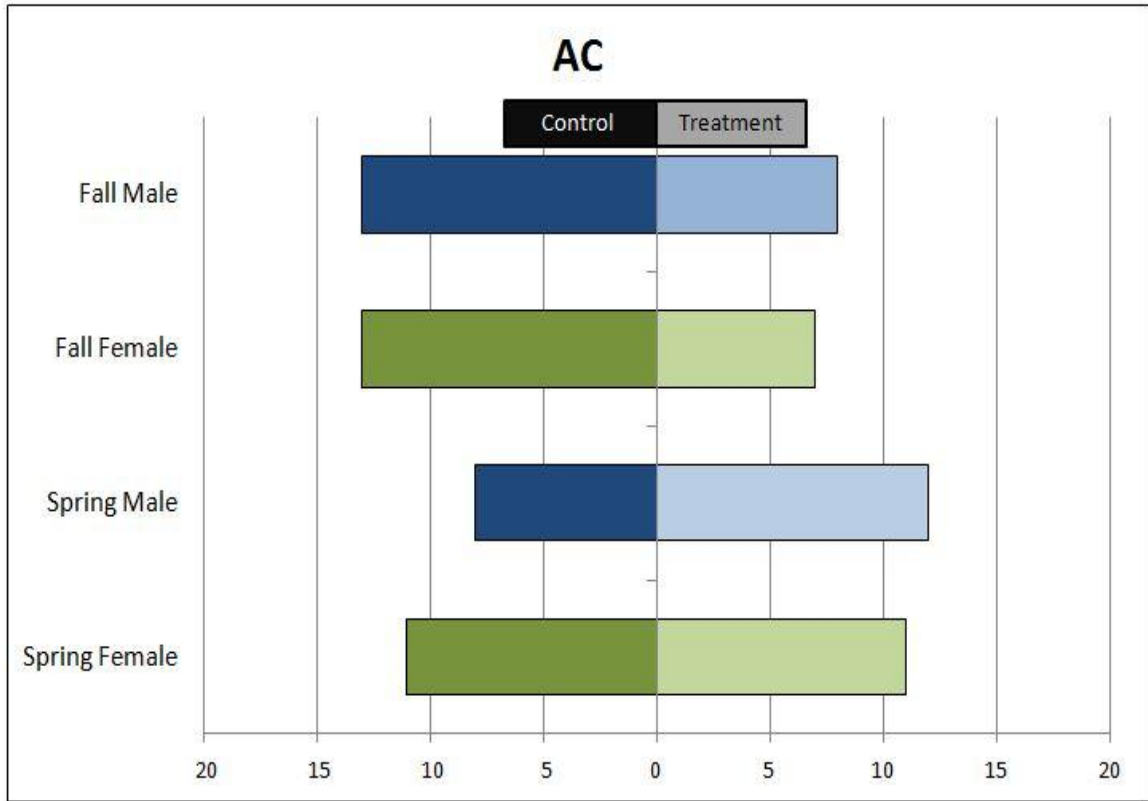


**Figure 31.** Y-tube olfactometer responses of female *C. sayi* towards each mixture of compounds regardless of season. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.

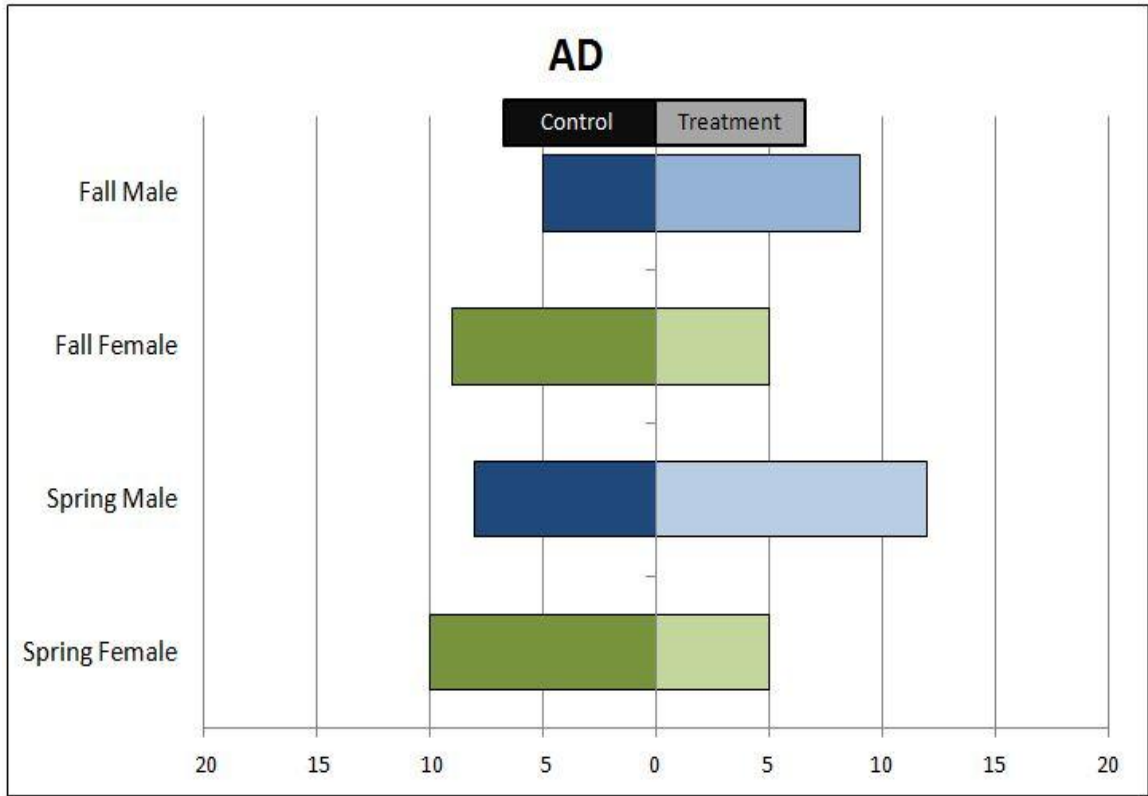


**Figure 32.** Y-tube olfactometer responses of male *C. sayi* towards each mixture of compounds regardless of season. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.

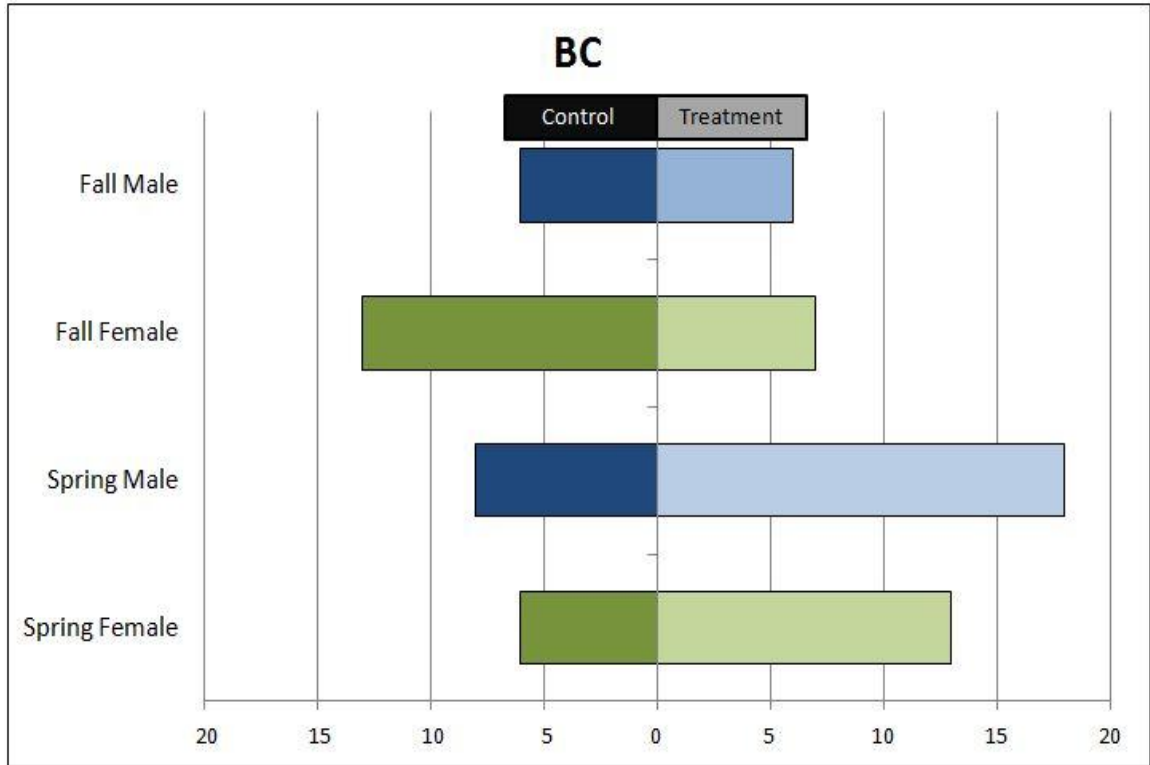




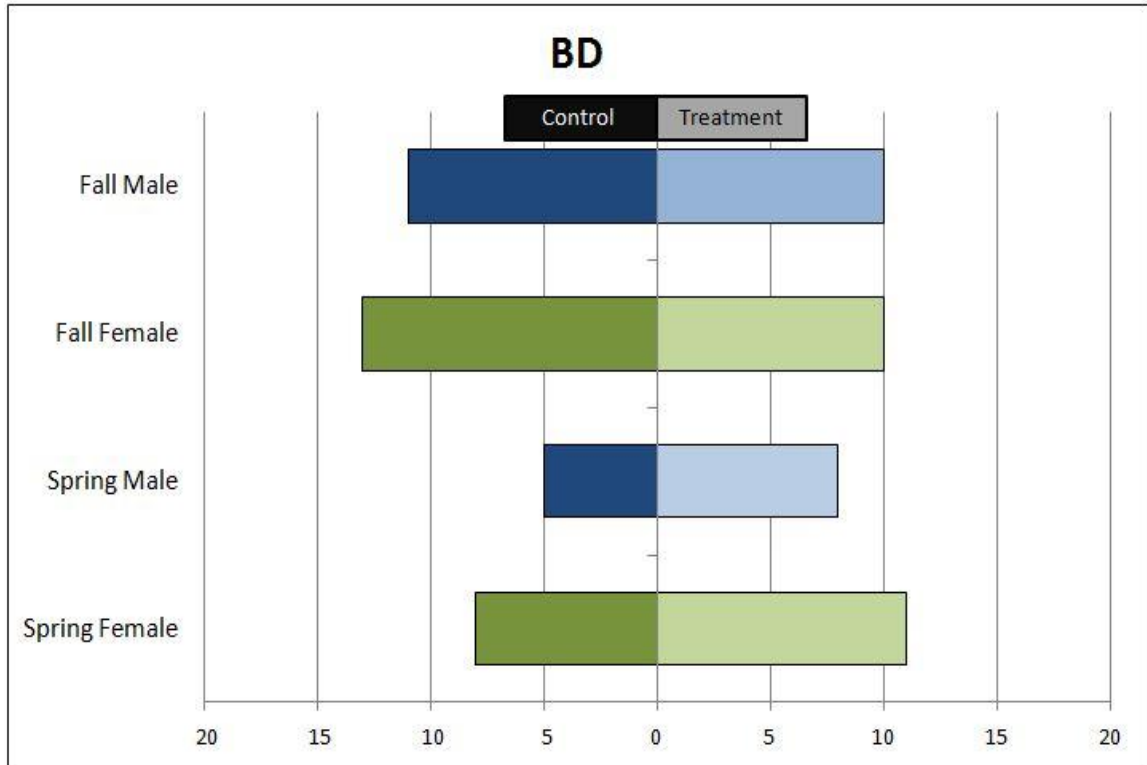
**Figure 33.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of activity towards a mixture of compounds (E)-2-hexenol and 2-heptanone. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



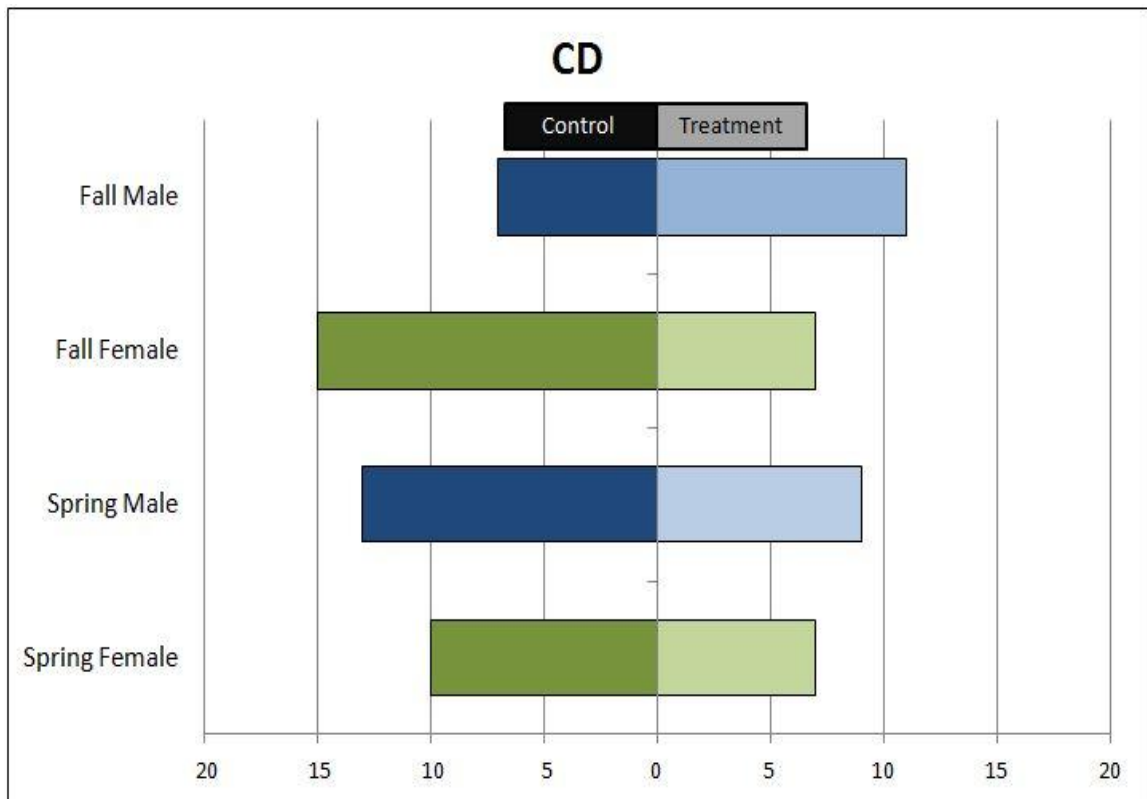
**Figure 34.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of activity towards a mixture of compounds (E)-2-hexenol and ethyl butyrate. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



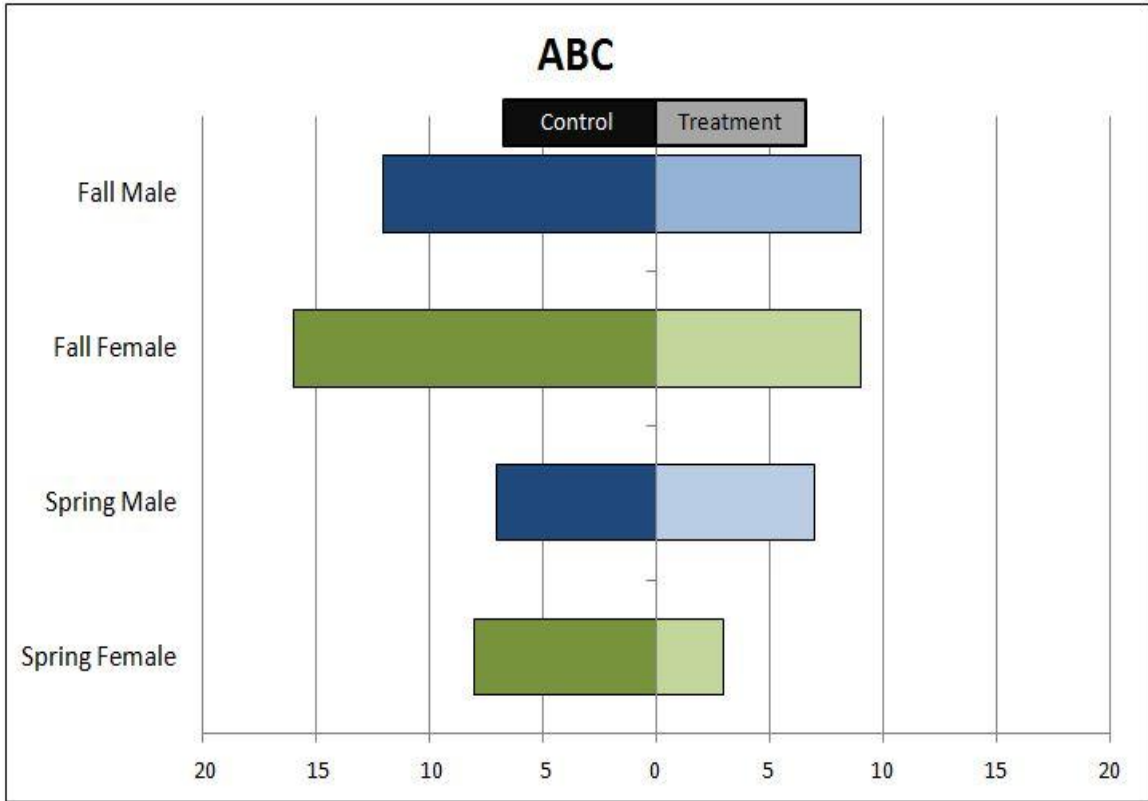
**Figure 35.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of activity towards a mixture of compounds (E)-2-hexenal and 2-heptanone. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



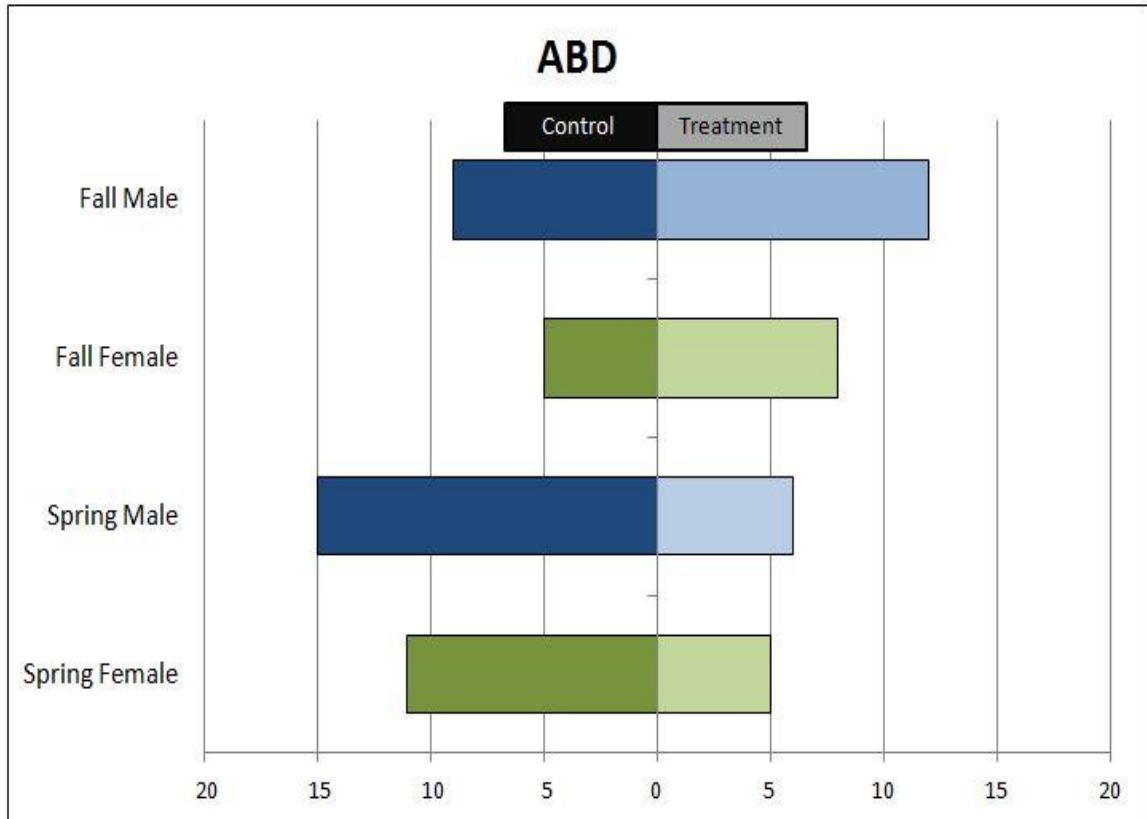
**Figure 36.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of activity towards a mixture of compounds (E)-2-hexenal and ethyl butyrate. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



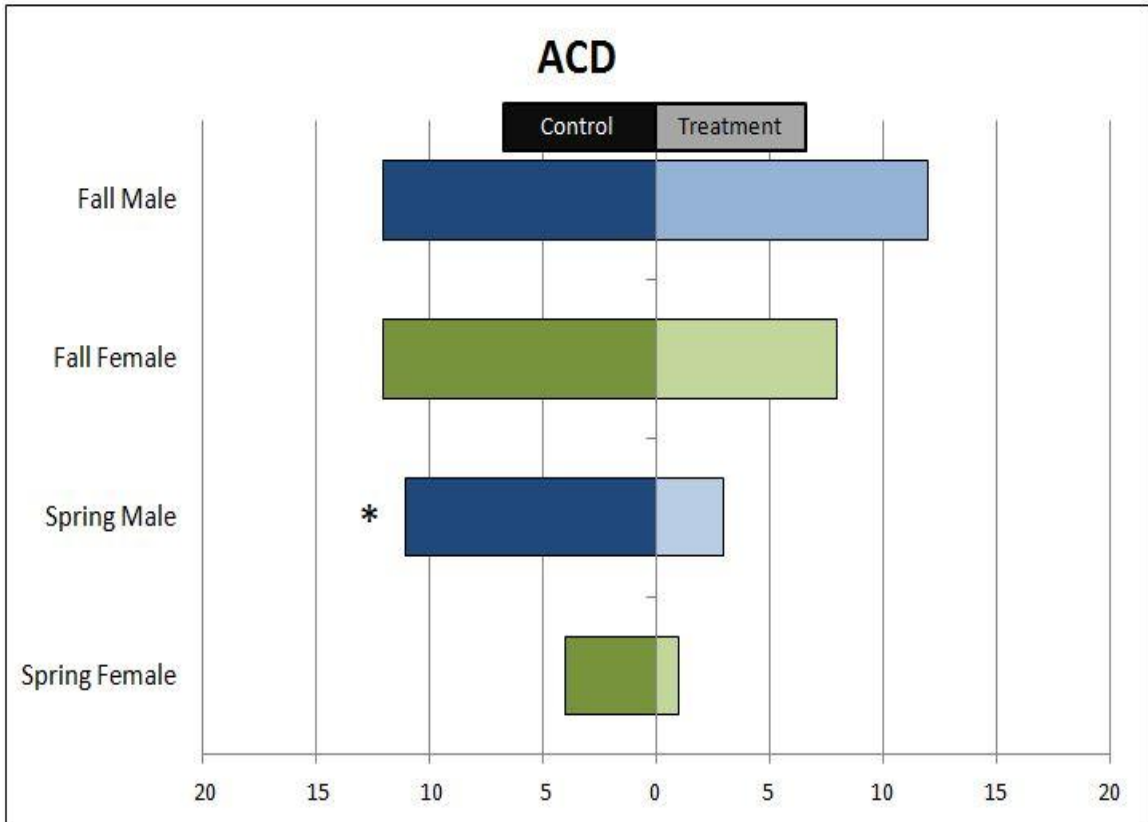
**Figure 37.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of activity towards a mixture of compounds 2-heptanone and ethyl butyrate. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



**Figure 38.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of activity towards a mixture of compounds (E)-2-hexenol, (E)-2-hexenal, and 2-heptanone. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.

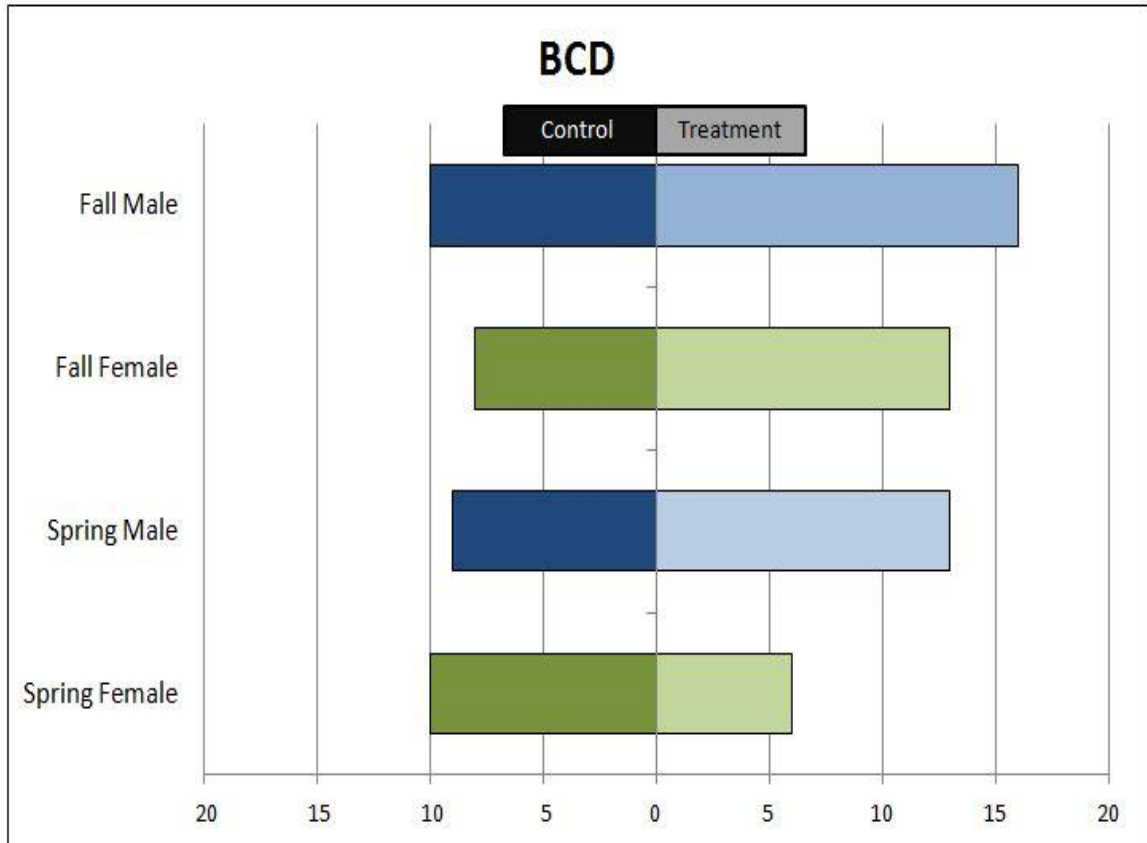


**Figure 39.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of activity towards a mixture of compounds (E)-2-hexenol, (E)-2-hexenal, and ethyl butyrate. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.

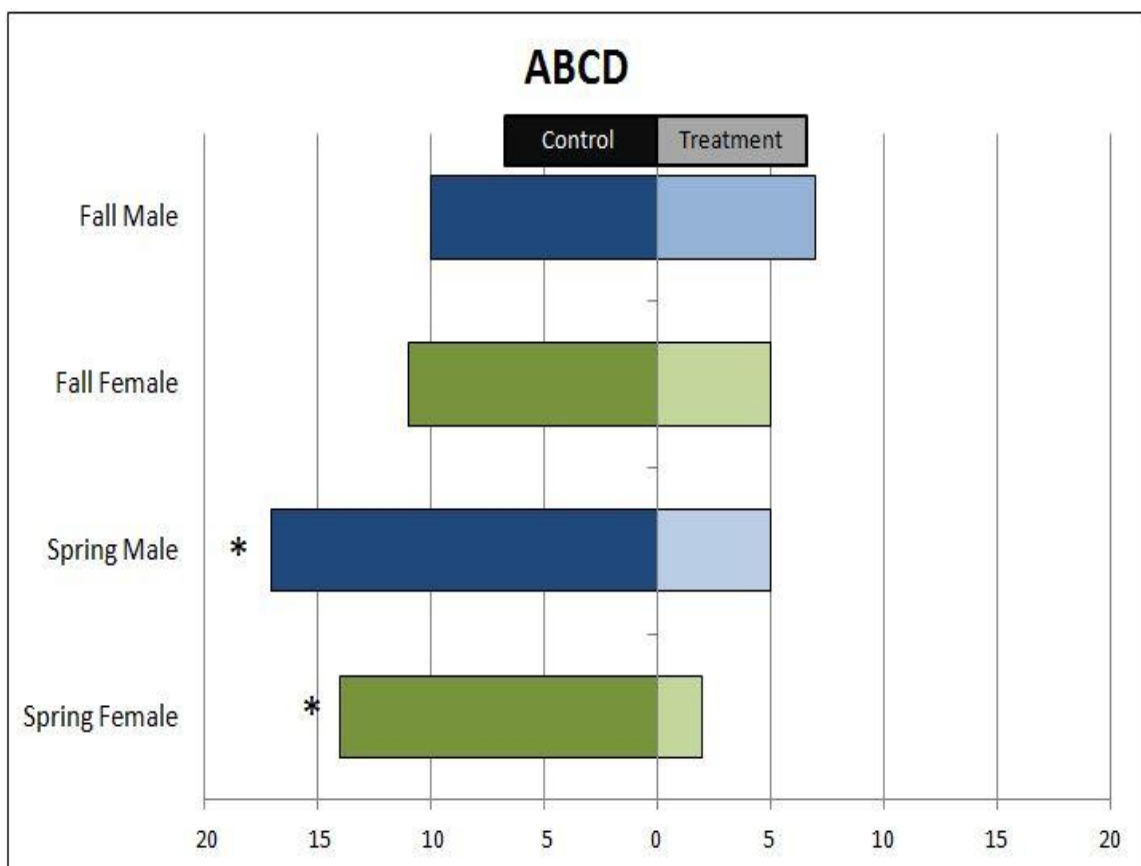


**Figure 40.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of activity towards a mixture of compounds (E)-2-hexenol, 2-heptanone, and ethyl butyrate. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.





**Figure 41.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of activity towards a mixture of compounds (E)-2-hexenol, 2-heptanone, and ethyl butyrate. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.



**Figure 42.** Y-tube olfactometer responses of *C. sayi* per sex and seasonal period of activity towards a mixture of compounds (E)-2-hexenol, (E)-2-hexenal, 2-heptanone, and ethyl butyrate. Presence of an asterisk indicates a significant difference ( $P < 0.05$ ) between the control and treatment responses.

## CHAPTER VI

### SUMMARY AND CONCLUSIONS

#### Physiological Responses

The physiological responses of adult *C. sayi* were consistent across both objectives with many significant responses to the selected volatile organic compounds (VOCs). The dose-response trials of (E)-2-hexenol, 2-heptanol, (E)-2-hexenal, 2-heptanone, ethyl butyrate, ethyl-2-methyl butyrate, ethyl tiglate, and ethyl isobutyrate provided a threshold for the concentration at which each of the compounds was detected. Additionally, when comparing spring-active to fall-active weevils there seemed to be a loss in sensitivity to the VOCs. Adult *C. sayi* collected in the fall did not respond significantly at the low doses of several of the VOCs like spring-active weevils did. Also fall-active males were more strongly affected than females; fall-active males did not respond significantly to all compounds at the 1:10 dilution like all other groups of weevils did, and when they did respond it was concentrated on the highest doses. Despite fewer significant responses at the two low doses from fall weevils, some of the responses that were significant were particularly large. The largest response from spring weevils regardless of sex, dose, or compound was only 65.12% from males at the 1:100 dilution of (E)-2-hexenol. At the 1:10 dilution, fall-active female responses were nearly twice as large at 158.09%, 135.47%, and 137.73% for 2-heptanone, ethyl-2-methyl butyrate, and ethyl tiglate, respectively. The changing of the EAG protocol between spring and fall though must be considered especially when it comes to the larger fall EAG responses. While the 1:10 and 1:1,000 dilutions in the spring weevils may have been affected by

antennal decay, it should be mitigated by the measurement of responses as a percentage of controls.

Ideally, the four dilutions would show the lowest concentration at which each compound was detectable along with the concentration when the weevils' responses level off. The initial hypothesis was that for a given compound adult *C. sayi* would not respond to the 1:10,000 dilution, but would respond to the other dilutions. The expected antennal responses would then differ by a factor of 10 between the middle two doses but then level off at the highest dose. The results however, generally showed that weevils responded only to the 1:10 and 1:100 doses making it more difficult to determine at what concentration antennal responses begin to level off. The exceptions to this rule were (E)-2-hexenol and (E)-2-hexenal, which both received responses to lower doses. Spring-active males responded to (E)-2-hexenol at the 1:10, 1:100, and 1:10,000 dilutions nearly equally, but most other similar responses were between the 1:10 and 1:100 doses. Along with (E)-2-hexenol and (E)-2-hexenal, equal responses were present in antennal responses to 2-heptanone, ethyl-2-methyl butyrate, and ethyl isobutyrate but across different groups of weevils. The 1:10 dilution was selected for further study after considering the unclear maximum responses and the consistent response of adult *C. sayi* to all compounds at the 1:10 dilution except ethyl-2-methyl butyrate and ethyl isobutyrate. At the 1:10 dilution, (E)-2-hexenol, (E)-2-hexenal, 2-heptanone, and ethyl butyrate were selected for further testing in mixtures. Insects identify host odors by interpreting the ratios of a conserved group of VOCs and often interactions between VOCs are responsible for behavioral responses.

Mixtures of (E)-2-hexenol, (E)-2-hexenal, 2-heptanone, and ethyl butyrate were examined in each possible combination of two, three and four VOCs. Although individual compounds were not tested in 2013, the dose-response data from 2012 can provide estimates for the expected additive antennal responses of each mixture of VOCs for comparison. Interactions were examined by considering both the approximations of the expected results from the dose-response data and the comparisons between mixtures. Positive and negative interactions among both fall-active weevils and female weevils followed a pattern of stronger synergistic effects and weaker interfering effects when compared to spring-active weevils and male weevils. The composition of the VOC mixtures and number of VOCs in a mixture also played a major role in the antennal responses. The strongest physiological responses were to each of the two-compound mixtures except for treatment AB ((E)-2-hexenol + (E)-2-hexenal). Among every group of weevils, treatment AB received the smallest responses and only a significant response from spring-active females. The similarity in chemical structure between (E)-2-hexenol and (E)-2-hexenal could be responsible for the non-significant responses from the AB treatment in comparison to the other two-compound mixtures. The comparison of the other two-compound mixtures to the three and four-compound mixtures suggests that the addition of more compounds may interfere with each other.

The strongest antennal response, regardless of sex or season, was to treatment CD (2-heptanone + ethyl butyrate) followed by treatments BC ((E)-2-hexenal + 2-heptanone) and BD ((E)-2-hexenal + ethyl butyrate). The four-compound mixture and the three-compound mixtures that included both 2-heptanone and ethyl butyrate received larger responses than the remaining VOC mixtures. Considering the strength of specific

compounds along with interference between structurally similar VOCs, further testing should focus on a limited number of diverse and physiologically active compounds. The physiological responses of adult *C. sayi* suggest that the behavioral responses of weevils will likely be strong for the two-compound mixtures and that mixtures containing both 2-heptanone and ethyl butyrate will receive stronger responses than other compounds. Additionally, season of activity and sex of the weevil should be considered for further study of adult *C. sayi*.

### **Behavioral Responses**

The behavioral responses of adult *C. sayi* were not as consistent as the physiological responses but both years of data provided several significant responses. The dose-response data tested (E)-2-hexenol, 2-heptanol, (E)-2-hexenal, 2-heptanone, ethyl butyrate, ethyl-2-methyl butyrate, ethyl tiglate, and ethyl isobutyrate at four dilutions: 1:10, 1:100, 1:1,000, and 1:10,000. The significant responses were among several groups of weevils, doses, and VOCs. Adult *C. sayi* responded to (E)-2-hexenol, 2-heptanol, (E)-2-hexenal, 2-heptanone, and ethyl-2-methyl butyrate. The spread of the responses was inconsistent with many responses at concentrations to which weevils showed no physiological response (Chapter II). The comparison to physiological responses along with the low number of repetitions suggests that chance played a role in the significance of behavioral responses. The behavioral response to (E)-2-hexenol at the highest dose from females however agrees with the physiological results. Females responded both behaviorally and physiologically to (E)-2-hexenol at the 1:10 dose but the behavioral response was repellent. This repellent response might match the initial hypothesis that at the highest dose weevils would be overwhelmed by the VOC and

repelled by it. More repetitions after the consideration of the physiological dose-responses could provide more potential attractants. Often attraction is the result of combining multiple VOCs so (E)-2-hexenol, (E)-2-hexenal, 2-heptanone, and ethyl butyrate were selected for further study.

The analysis of multiple VOCs combined provided more consistent behavioral responses but did not necessarily agree with the physiological results to the same VOC combinations. The physiological responses were larger for the two compound mixtures and particularly those involving 2-heptanone and ethyl butyrate while the behavioral responses did not follow a similar pattern. The mixture of (E)-2-hexenol, 2-heptanone, and ethyl butyrate along with the mixture of all four VOCs received significant responses from spring-active male and spring-weevils respectively. Spring-active, female, and male weevils received significant behavioral responses when considered individually with the VOC mixtures. Each combined group of weevils responded to the four-compound mixture by avoiding the treatment odor. The consistent negative response to all the VOCs is just further confirmation that care is required in the selection and combination of selected VOCs if attraction is the goal. The only response that was attractive among any group of weevils was treatment BC ((E)-2-hexenal + 2-heptanone) to spring-active weevils. Both physiologically and behaviorally the combination of (E)-2-hexenal and 2-heptanone provided significant responses and could be a possible field attractant.

## REFERENCES CITED

- The American Chestnut Foundation. (Restoring the American Chestnut) 2007. Restoring the American Chestnut. ([http://www.acf.org/mission\\_history.php](http://www.acf.org/mission_history.php)).
- Addesso, K. M., H. J. McAuslane, and H. T. Alborn. 2011. Attraction of pepper weevil to volatiles from damaged pepper plants. *Entomologia Experimentalis et Applicata* 138: 1–11.
- Alm, S. R., and F. R. Hall. 1986. Antennal sensory structures of *Conotrachelus nenuphar* (Coleoptera: Curculionidae). *Annals of the Entomological Society of America* 79: 324–333.
- Altuzar, A., E. A. Malo, H. Gonzalez-Hernandez, and J. C. Rojas. 2007. Electrophysiological and behavioural responses of *Scyphophorus acupunctatus* (Coleoptera: Curculionidae) to *Agave tequilana* volatiles. *Journal of Applied Entomology* 131: 121–127.
- Anagnostakis, S. L. 1987. Chestnut Blight: The classical problem of an introduced pathogen. *Mycologia* 79: 23–37.
- Baker, T. C., and R. T. Cardé. 1984. Techniques for Behavioral Bioassays, pp. 45–73. *In* Hummel, H.E., Miller, T.A. (eds.), *Techniques in Pheromone Research*, Springer Series in Experimental Entomology. New York.
- Barakat, A., D. S. DiLoreto, Y. Zhang, C. Smith, K. Baier, W. A. Powell, N. Wheeler, R. Sederoff, and J. E. Carlson. 2009. Comparison of the transcriptomes of American chestnut (*Castanea dentata*) and Chinese chestnut (*Castanea mollissima*) in response to the chestnut blight infection. *BMC Plant Biology* 9: 51.
- Baxter, D. V., and L. S. Gill. 1931. Deterioration of chestnut in the southern Appalachians. No. 257. U.S. Department of Agriculture.
- Bland, R. G. 1983. Sensilla on the antennae, mouthparts, and body of the larva of the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera : Curculionidae). *International Journal of Insect Morphology and Embryology*. 12: 261–272.
- Brooks, F. E., and R. T. Cotton. 1929. The chestnut curculios. U.S. Government Printing Office, Technical Bulletin No. 130.
- Bruce, T. J. A., L. J. Wadhams, and C. M. Woodcock. 2005. Insect host location: a volatile situation. *Trends in Plant Science*. 10: 269–274.
- Buttrick, P. 1925. Chestnut in North Carolina. Chestnut and the chestnut blight in North Carolina. 6–10.



- Cooper, W. R., and L. K. Rieske. 2010. Gall structure affects ecological associations of *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae). *Environmental Entomology* 39: 787–797.
- Cooper, W., and L. Rieske. 2009. Woody stem galls interact with foliage to affect community associations. *Environmental Entomology* 38: 417–424.
- Dickens, J. C. 1989. Green leaf volatiles enhance aggregation pheromone of boll weevil, *Anthonomus grandis*. *Entomologia Experimentalis et Applicata* 52: 191–203.
- Gold, M. A., M. M. Cernusca, and L. D. Godsey. 2005. Chestnut market analysis producer's prospective. University of Missouri Center for Agroforestry.
- Gold, M. A., M. M. Cernusca, and L. D. Godsey. 2006. Competitive market analysis: chestnut producers. *Horticultural Technology* 16: 360–369.
- Griffin, G. J. 2000. Blight control and restoration of the American chestnut. *Journal of Forestry* 98: 22–27.
- Guarino, S., P. L. Bue, E. Peri, and S. Colazza. 2011. Responses of *Rhynchophorus ferrugineus* adults to selected synthetic palm esters: electroantennographic studies and trap catches in an urban environment. *Pest Management Science* 67: 77–81.
- Gunawardena, N. E., and S. Dissanayake. 2000. Host attractants for the banana stem borer, *Odoiporus longicollis* (Coleoptera: Curculionidae): identification, electrophysiological activity and behavioural bioassay. *Journal of the National Science Foundation of Sri Lanka* 28: 231–242.
- Hansson, B. S. 2002. A bug's smell – research into insect olfaction. *Trends in Neurosciences* 25: 270–274.
- Hansson, B. S., M. C. Larsson, and W. S. Leal. 1999. Green leaf volatile-detecting olfactory receptor neurones display very high sensitivity and specificity in a scarab beetle. *Physiological Entomology* 24: 121–126.
- Hebard, F. V. 1982. Biology of virulent and hypovirulent *Endothia parasitica* on American chestnut (*Castanea dentata*) (PhD Dissertation). Virginia Polytechnic Institute and State University.
- Hibbard, B. E., T. L. Randolph, E. J. Bernklau, E. M. Abou-fahr, and L. B. Bjostad. 1997. Electroantennogram-active components of maize silk for adults of the western corn rootworm (Coleoptera: Chrysomelidae). *Environmental Entomology* 26: 285–296.
- Hillman, B. I., and N. Suzuki. 2004. Viruses of the Chestnut Blight Fungus, *Cryphonectria parasitica*, pp. 423–472. *In Advances in Virus Research*. Academic Press.

- Hughes, J., and A. P. Vogler. 2004. The phylogeny of acorn weevils (genus *Curculio*) from mitochondrial and nuclear DNA sequences: the problem of incomplete data. *Molecular Phylogenetics and Evolution* 32: 601–615.
- Hunt, K., M. A. Gold, W. Reid, and M. R. Warmund. 2009. Growing Chinese chestnuts in Missouri. *Agroforestry in Action AF 1007*. University of Missouri Center for Agroforestry. Columbia, Missouri.
- Johnson, W. T. 1950. On the biology and control of the North American chestnut weevils (PhD Dissertation). University of Maryland.
- Jönsson, M., and P. Anderson. 1999. Electrophysiological response to herbivore-induced host plant volatiles in the moth *Spodoptera littoralis*. *Physiological Entomology* 24: 377–385.
- Kaissling, K. E. 1986. Peripheral mechanisms of pheromone reception in moths. *Chemical Senses* 21: 256–268.
- Keesey, I. W. 2011. The chemical ecology of the lesser chestnut weevil: behavioral and electrophysiological responses of *Curculio sayi* (Coleoptera: Curculionidae) to host-plant volatile organic compounds (PhD Dissertation). University of Missouri.
- Keesey, I. W., and B. A. Barrett. 2008. Seasonal occurrence and soil distribution of the lesser chestnut weevil, *Curculio sayi* (Coleoptera: Curculionidae) in Mid-Missouri. *Journal of the Kansas Entomological Society* 81: 345–354.
- Keesey, I. W., and B. A. Barrett. 2012. Behavioral and electroantennographic responses of the lesser chestnut weevil, *Curculio sayi* (Coleoptera: Curculionidae), to odors emanating from different chestnut plant tissues. *Journal of the Kansas Entomological Society* 85: 145–154.
- Keesey, I. W., B. A. Barrett, C.H. Lin, and R. N. Lerch. 2012. Electroantennographic responses of the small chestnut weevil *Curculio sayi* (Coleoptera: Curculionidae) to volatile organic compounds identified from chestnut reproductive plant tissue. *Environmental Entomology* 41: 933–940.
- Kozłowski, M. W., and J. H. Visser. 1981. Host plant related properties of the antennal olfactory system in the oak flea weevil, *Rhynchaenus quercus*. electroantennogram study. *Entomologia Experimentalis et Applicata* 30: 169–175.
- Lang, P., F. Dane, and T. L. Kubisiak. 2006. Phylogeny of *Castanea* (Fagaceae) based on chloroplast trnT-L-F sequence data. *Tree Genetics & Genomes* 2: 132–139.
- Lang, P., F. Dane, T. L. Kubisiak, and H. Huang. 2007. Molecular evidence for an Asian origin and a unique westward migration of species in the genus *Castanea* via Europe to North America. *Molecular Phylogenetics and Evolution* 43: 49–59.

- Leskey, T. C., R. J. Prokopy, S. E. Wright, P. L. Phelan, and L. W. Haynes. 2001. Evaluation of individual components of plum odor as potential attractants for adult plum curculios. *Journal of Chemical Ecology* 27: 1–17.
- Leskey, T. C., S. E. Wright, W. Anger, G. Chouinard, D. Cormier, A. Pichette, and A. Zhang. 2009. Electroantennogram technique for *Conotrachelus nenuphar* (Coleoptera: Curculionidae). *Environmental Entomology* 38: 870–878.
- Light, D. M., R. A. Flath, R. G. Buttery, F. G. Zalom, R. E. Rice, J. C. Dickens, and E. B. Jang. 1993. Host-plant green-leaf volatiles synergize the synthetic sex pheromones of the corn earworm and codling moth (Lepidoptera). *Chemoecology*. 4: 145–152.
- Merkel, H. 1905. A deadly fungus on the American chestnut. New York Zoological Society. 10th Annual Report: 97–103.
- Murrill, W. A. 1906. A serious chestnut disease. *New York Botanical Garden* 7: 203–211.
- Payne, J. A., R. A. Jaynes, and S. J. Kays. 1983. Chinese chestnut production in the United States: practice, problems, and possible solutions. *Economic Botany* 37: 187–200.
- Piñero, J. C., and R. J. Prokopy. 2003. Field evaluation of plant odor and pheromonal combinations for attracting plum curculios. *Journal of Chemical Ecology* 29: 2735–2748.
- Piñero, J. C., S. E. Wright, and R. J. Prokopy. 2001. Response of plum curculio (Coleoptera: Curculionidae) to odor-baited traps near woods. *Journal of Economic Entomology* 94: 1386–1397.
- Roelofs, W. L. 1984. Electroantennogram Assays: Rapid and convenient screening procedures for pheromones, pp. 131–159. *In* Hummel, H.E., Miller, T.A. (eds.), *Techniques in Pheromone Research*, Springer Series in Experimental Entomology. New York.
- Russell, E. W. B. 1987. Pre-Blight distribution of *Castanea dentata* (Marsh.) Borkh. *Bulletin of the Torrey Botanical Club* 114: 183–190.
- Säid, I., D. Tauban, M. Renou, K. Mori, and D. Rochat. 2003. Structure and function of the antennal sensilla of the palm weevil *Rhynchophorus palmarum* (Coleoptera, Curculionidae). *Journal of Insect Physiology* 49: 857–872.
- SAS Institute. 2011. Version 9.3. SAS Institute Inc., Cary, North Carolina.
- Schneider, D. 1957. Electrophysiological investigation on the antennal receptors of the silk moth during chemical and mechanical stimulation. *Experientia* 13: 89–91.

- Schneider, D., and U. Seibt. 1969. Sex pheromone of the queen butterfly: electroantennogram responses. *Science* 164: 1173–1174.
- Senter, S. D., J. A. Payne, G. Miller, and S. L. Anagnostakis. 1994. Comparison of total lipids, fatty acids, sugars and nonvolatile organic acids in nuts from four *Castanea* species. *Journal of the Science of Food and Agriculture* 65: 223–227.
- Stelinski, L. L., J. R. Miller, and L. J. Gut. 2003. Presence of long-lasting peripheral adaptation in oblique-banded leafroller, *Choristoneura rosaceana* and absence of such adaptation in redbanded leafroller, *Argyrotaenia velutinana*. *Journal of Chemical Ecology* 29: 405–423.
- Struble, D. L., and H. Arn. 1984. Combined gas chromatography and electroantennogram recording of insect olfactory responses, pp. 161–178. *In* Hummel, H.E., Miller, T.A. (eds.), *Techniques in Pheromone Research*, Springer Series in Experimental Entomology. New York.
- Sullivan, B. T. 2005. Electrophysiological and behavioral responses of *Dendroctonus frontalis* (Coleoptera: Curculionidae) to volatiles isolated from conspecifics. *Journal of Economic Entomology* 98: 2067–2078.
- Sun, X.-L., G.-C. Wang, X.-M. Cai, S. Jin, Y. Gao, and Z.-M. Chen. 2010. The tea weevil, *Myloccerinus aurolineatus*, is attracted to volatiles induced by conspecifics. *Journal of Chemical Ecology* 36: 388–395.
- Szendrei, Z., E. Malo, L. Stelinski, and C. Rodriguez-Saona. 2009. Response of cranberry weevil (Coleoptera: Curculionidae) to host plant volatiles. *Environmental Entomology* 38: 861–869.
- Van Tol, R. W. H. M., D. J. Bruck, F. C. Griepink, and W. J. De Kogel. 2012. Field attraction of the vine weevil *Otiiorhynchus sulcatus* to kairomones. *Journal of Economic Entomology* 105: 169–175.
- Van Tol, R. W. H. M., J. H. Visser, and M. W. Sabelis. 2002. Olfactory responses of the vine weevil, *Otiiorhynchus sulcatus*, to tree odours. *Physiological Entomology* 27: 213–222.
- Toshova, T. B., D. I. Velchev, M. A. Subchev, M. Tóth, J. Vuts, J. A. Pickett, and S. Y. Dewhirst. 2010. Electrophysiological responses and field attraction of the grey corn weevil, *Tanymecus (Episomecus) dilaticollis* Gyllenhal (Coleoptera: Curculionidae) to synthetic plant volatiles. *Chemoecology* 20: 199–206.
- Visser, J. H. 1986. Host odor perception in phytophagous insects. *Annual Review of Entomology* 31: 121–144.
- Visser, J. H. 1988. Host-plant finding by insects: orientation, sensory input and search patterns. *Journal of Insect Physiology* 34: 259–268.

- Wee, S.-L., A. M. El-Sayed, A. R. Gibb, V. Mitchell, and D. M. Suckling. 2008. Behavioural and electrophysiological responses of *Pantomorus cervinus* (Boheman) (Coleoptera: Curculionidae) to host plant volatiles. *Australian Journal of Entomology* 47: 24–31.
- Woodruff, J. B. 1946. Chestnut blight in Italy. *Trees (Journal of American Arboriculture)* April. 16, 8-9.