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Chemical Ecology of Click Beetles (Coleoptera: Elateridae): First Identification of Sex Pheromones and Sex Attractants of North American Species

A Dissertation submitted in partial satisfaction
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by

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ABSTRACT OF THE DISSERTATION

Chemical Ecology of Click Beetles (Coleoptera: Elateridae): The Identification of Sex Pheromones and Sex Attractants of North American Species

by

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Click beetles (Coleoptera: Elateridae) are one of the most diverse and well recognized groups of beetles worldwide. Their diverse habitats and feeding behaviors result in a wide variety of ecological and economic roles for click beetles. Wireworms, the term applied to larvae of pestiferous species, form a devastating pest complex that threatens numerous vegetable and cereal crops on all continents. To date, the pheromonal signals that are crucial mediators of reproductive behaviors have not been exploited in integrated pest management (IPM) programs for North American elaterids. The utility of such pheromones for monitoring and control has been demonstrated for Eurasian elaterids, but prior to my thesis research, pheromones had not been identified for any North American species. Thus, my main goals were to jump-start elaterid pheromone research in North America, and to develop efficient protocols for collection, identification, and bioassay of elaterid pheromones.

The first portion of this work describes the identification of sex pheromones, sex attractants, and a hyperpheromone for two sympatric *Cardiophorus* species. Males were initially attracted to a known cerambycid beetle pheromone, fuscumol acetate. The actual pheromone was subsequently identified as (*R*)-methyl dihydrofarnesoate, which is structurally similar to fuscumol acetate. In a follow-up study, additional pheromone analogs were tested to further investigate the specificity of responses of males, resulting in the discovery of a compound which was >26 times as attractive as the natural pheromone of *Cardiophorus edwardsi*.

In the second section, I identified 13-tetradecenyl acetate as the sex pheromomone of *Melanotus communis*. Additional studies optimized lures for use in IPM programs for this agricultural pest. The final section describes identification of the sex pheromone of *Idolus californicus,* which started from a lead obtained from field screening of known pheromones of European species. Males were specifically attracted to neryl hexanoate, one of two compounds which were subsequently identified from females. The second compound was not attractive, although it elicited antennal responses from males, and also attracted males of a *Dalopius* species. The work presented here should lay the groundwork for future research into elaterid pheromones across the United States and worldwide.

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Chapter 1: Introduction

Click beetles (Coleoptera: Elateridae) or elaterids, are one of the 10 largest beetle families, with ~10,000 described species worldwide (Bouchard et al. 2017). Elateridae is a cosmopolitan beetle family, and click beetles are some of the most abundant beetles in terrestrial habitats (Douglas 2011). Elaterids are well known for their audible clicking mechanism, which is a rapid movement of a thoracic hinge that is present between the prothorax and mesothorax (Evans 1972). This mechanism is used by the beetles as a means to right themselves and to escape or startle a predator (Evans and Hogue 2006).

Adults of most click beetle species can fly (Johnson 2002) and can be found on vegetation or underneath tree bark (Johnson 2002; Evans and Hogue 2006). Not all species of click beetles feed as adults, but those that do exhibit a wide range of feeding behaviors such as herbivory (e.g. fruit, nectar, pollen, etc.) and predation on honey-dew producing hemipterans (Johnson 2002; Evans and Hogue 2006). Voltinism varies within the family (Furlan 1996; Vernon and van Herk 2013; Vernon and van Herk 2014). Despite the relatively large number of species, and the economic importance of some species, there is surprisingly little information on their reproductive biology.

Elaterid larvae can be found in the soil or decaying plant material such as leaves or wood, and like the adults, there is a wide range of larval feeding habits within the family (Johnson 2002; Evans and Hogue 2006; Traugott et al. 2015). Wood-inhabiting larvae can be saprophagous or predaceous on small invertebrates (Johnson 2002; Evans and Hogue 2006), and can be important for the biological control of forest pests (Morris 1951; Turnock 1968; Zhang et al. 2008). The larvae of species that are agricultural pests,

also known as wireworms, can be some of the most damaging pests of field crops. This is due to the fact that wireworms can feed on seedlings, roots, and tubers of a variety of crops such as cereals (i.e. wheat, corn, and barley) and root vegetables (i.e. potatoes, beets, and carrots) (Johnson 2002; Evans and Hogue 2006; Traugott et al. 2015). Damage to these portions of the plant can significantly reduce yields as well as increasing exposure to pathogens (Keiser et al. 2012), decreasing growth, and causing cosmetic damage (Traugott et al. 2015).

Identification of larval and adult click beetles is challenging because their morphologically based taxonomy is complex, and relationships within and between some taxonomic groups are not clearly established yet. Wireworms can often be keyed to the genus level without much difficulty, however some genera and species (e.g. *Agriotes*, *Melanotus*, *Hypnoidus*, and *Hadromorphus*) are virtually impossible to differentiate on the basis of morphological characters alone (Etzler et al. 2014; Traugott et al. 2015). To add another level of complexity, it is known that there are cryptic species and species complexes in both agricultural and forest environments (e.g., *Melanotus communis* and *Idolus* spp.) (Tolasch et al. 2013; Etzler et al. 2014; König et al. 2015).

Economic importance of elaterids, and management methods

Wireworms have been key pests in agriculture for centuries because they are a threat to many major crops globally (Traugott et al. 2015). The development of synthetic insecticides (e.g. carbamates, organophosphates, and organochlorines) in the last half of the 20th century changed the whole dynamic of wireworm management. Organochlorine

compounds (OCs) were among the most popular insecticides used to control wireworms, and were favored for their long term effectiveness and persistence in the soil. The effectiveness of these and other insecticides used to control wireworms curbed the development of additional control strategies until the 1990s (Parker 2005) and resulted in wireworms being treated as a single pest entity.

However, during the last two decades of the 20th century, many of the highly effective OCs were withdrawn from registration because of environmental and health concerns which then exacerbated the need to develop alternative methods of managing wireworms (Parker 2005). Before the turn of the century, insecticidal control of wireworms was still mostly dependent on less effective and persistent organophosphates (OPs) and carbamates (CAs) (Traugott et al. 2015). In the 2000s, newer classes of insecticides (e.g. pyrethroids and neonicotinoids) were introduced for wireworm management but some were ineffective (Vernon et al. 2009; Vernon et al. 2013; Esser et al. 2015; Van Herk et al. 2015; Larsen et al. 2016; Langdon et al. 2018; Herk et al. 2019). Overall, effective chemical options for in-season control of wireworms are disappearing and as a result, populations in agricultural crops will increase.

Currently, soil cores and bait stations are the two main methods used to sample wireworms in the soil. Soil cores can be used to estimate wireworm abundance before planting and during a growing season. These methods are very labor intensive and unlikely to give reliable estimates of abundance due to larvae having clumped distributions (Doane 1977; Williams et al. 1992; Cherry and Stansly 2008; Benefer et al. 2010), and variation in bait preference (Jansson and Lecrone 1989; Arakaki et al. 2009).

Bait stations are slightly more effective than soil cores for detecting wireworms (Parker 1994; Simmons et al. 1998) because wireworms are attracted to volatiles from germinating seed baits and roots (Doane et al. 1975; Gfeller et al. 2013; Barsics et al. 2014; Barsics et al. 2017).

Due to the difficulty in sampling and controlling wireworms, attention is now shifting to the possibility of monitoring and controlling the adult stage to limit oviposition in susceptible fields and nearby habitats (Vernon and Van herk 2013; Reddy and Tangtrakulwanich 2014; Traugott et al. 2015). Until the identification of sex pheromones, adult click beetles were sampled primarily with unbaited pitfall and sticky traps, which were of limited efficacy (Salt and Hollick 1944; Genung 1972; Furlan 1996).

Chemical Ecology of Click Beetles

Before 2018, only a few pheromones and pheromone candidates had been identified from the Elateridae, and all available data was from 34 species native to Europe and Asia (reviewed in Tóth 2013; Tolasch et al. 2013; König et al. 2015; König et al. 2016). The first successful identification of click beetle pheromones was reported by Yatsynin et al. (1980), where pheromones were identified from three species in the genus *Agriotes* Eschscholtz. The pheromones of *Agriotes gurgistanus* (Falderman), *A. littigiosus* Rossi, and *A. lineatus* L. were identified from extracts of pheromone glands (Yatsynin et al. 1980) located in the abdomens of female beetles (Ivastschenko and Adamenko 1980; Merivee and Erm 1993). The pheromones of *A. gurgistanus* and *A. littigiosus* were identified as geranyl butyrate [(*E*)-3,7-dimethyl-2,6-octadien-1-yl

butanoate] and geranyl isovalerate [(*E*)-3,7-dimethyl-2,6-octadien-1-yl 3-methylbutanoate], respectively. (*E,E*)-Farnesyl acetate [(2*E,*6*E*)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl acetate] and neryl isovalerate [(*Z*)-3,7-dimethylocta-2,6-dienyl 3-methylbutanoate] were identified as the pheromones of *A. lineatus.*

Since 1980, pheromones and pheromone candidates have been identified from several more species in the genus *Agriotes* (reviewed in Tóth 2013), most likely because *Agriotes* species are the most important elaterid pests in Europe. To date, a majority of elaterid pheromones and pheromone candidates have been identified from *Agriotes* species, but there have been some identified for a few species from the genera *Ampedus* Germar (Yatsynin et al. 1996), *Athous* Eschscholtz (Kamm et al. 1983)*, Betarmon* Kiesenwetter (König et al. 2016)*, Cidnops* (Tóth et al. 2003)*, Ectinus* Eschscholtz (Tolasch 2008)*, Elater* L. (Tolasch et al. 2007), *Idolus* Desbrochers des Loges (Tolasch et al. 2013; König et al. 2015), *Melanotus* Eschscholtz (Tamaki et al. 1986; Tamaki et al. 1990; Yatsynin et al. 1996; Yen and Chen 1998; Tolasch et al. 2007)*, Synaptus* Eschscholtz (Yatsynin et al. 1996).

All sex pheromones and pheromone candidates known to date have been identified from female beetles in species in the subfamily Elaterinae. Although the existing data are not extensive, patterns of conservation of pheromone structures within closely related taxa are beginning to emerge. For exampole, the pheromone compounds identified from *Elater ferrugineus* (Tolasch et al. 2007), *Ectinus aterrimus* (Tolasch 2008)*,* and a *Sericus* species (König et al. 2016), are 7-methyloctyl esters with even numbered acids, such as 7-methyloctyl octanoate. Analogously, *Melanotus* species (tribe:

Melanotini) from Japan produce dodecyl acetate and esters of a terminally conjugated alcohol [(*E*)-9,11-dodecadien-1-ol] (Tamaki et al. 1986; Tamaki et al. 1990; Yen and Chen 1998). A few *Melanotus* species from Europe were reported to produce saturated and unsaturated esters based on 12 and 14 carbon alcohols, such as tetradecenyl butyrate (Yatsynin et al. 1996; Tolasch et al. 2007), but structure identifications were incomplete (i.e. position and geometry of C=C double bonds) and there are no reports of field bioassays to confirm the activity of the pheromone candidates. Several species in the genus *Agriotes* produce esters of terpenoid alcohols such as geraniol, nerol, and farnesol (reviewed in Tóth 2013). Recently, pheromones have been identified from a few species in the genera *Idolus* (Tolasch et al. 2013; König et al. 2015) and *Betarmon* (König et al. 2016), and these species produce some of the same terpenoid esters as *Agriotes* species.

Much of the pheromone literature suggests that elaterids use female-produced sex pheromones, but there are some studies that suggest that the pheromones of some *Agriotes* species could in fact be aggregation pheromones which are attractive to both sexes (Tóth 2013; Tóth et al. 2015; Vuts et al. 2018). There are no other reports of female beetles being caught in pheromone-baited traps in the remaining literature on elaterid pheromones, but electroantennogram studies on *Agriotes brevis* and *A. sordidus* showed that antennae of females and males exhibited similar response intensities to pheromone components (Tóth 2013; Tóth et al. 2015).

In Japan, pheromones have been utilized for the monitoring and management of two pestiferous *Melanotus* species (Nagamine and Kinjo 1990; Kishita et al. 2003; Arakaki et al. 2008a, 2008b, 2008c). *Melanotus okinawensis* and *M. sakishimensis* larvae

are serious pests of sugarcane, feeding on underground buds which often results in germination failure. For *M. sakishimensis,* pheromone traps have been used to estimate population abundance and peak occurrences of adult males (Arakaki et al. 2008c). In addition to population density estimates (Kishita et al. 2003), pheromones of *M. okinawensis* show promise for mass trapping (Arakaki et al. 2008b) and mating disruption (Arakaki et al. 2008a).

In North America, there are ~1000 described species of elaterids (Bouchard et al. 2017). Prior to the work reported in this dissertation, there had been no successful pheromone identifications for any North American elaterids. Preliminary click beetle pheromone research in the United States, showed that, for several species, males were attracted to female beetles and/or extracts of females (Shirck 1942; Lilly 1959; Doane 1961; Chapman 1964; Lilly and McGinnis 1965; Jacobson et al. 1968; Hayes and Wheeler 1968; Onsager et al. 1968; Lilly and McGinnis 1968), suggesting that females were the pheromone-producing sex. The first pheromones to be identified for North American elaterids were valeric and caproic acid, as the pheromones for *Limonius californicus* (Mannerheim) (Jacobson et al. 1968) and *L. canus* L. (Butler et al. 1975), respectively. However, neither of these compounds attracted *L. californicus*, *L. canus,* or other species when they were field tested in several states (unpublished data, P. Landolt and D. Horton in WA, G. Reddy in MT), suggesting that these identifications were not correct.

Lastly, several reports had indicated that *Melanotus depressus* Melsheimer males were attracted to traps baited with the pheromone blend of the tufted apple budmoth

(*Platynota idaeusalis* Walker) (Weires 1976; Brown and Keaster 1983; Brown and Keaster 1986; Keaster et al. 1987), which consists of (*E*)-11-tetradecenyl acetate and (*E*)-11-tetradecen-1-ol (Hill et al. 1974). However, it was never confirmed if *Melanotus depressus* females actually produce (*E*)-11-tetradecenyl acetate and (*E*)-11-tetradecen-1-ol, and whether males are attracted to one or both these compounds.

Summary

In the 20th century, broad spectrum and highly persistent insecticides such as lindane (*gamma*-hexachlorocyclohexane) provided good control of wireworms, which discouraged the development of additional control strategies until the 1990s (Parker 2005), and resulted in wireworms being treated as a single pest entity. Since lindane was banned in the U.S. in 2009, there has been increased interest in investigating click beetle pheromones for possible practical applications. It has been suggested that pheromones could be developed for monitoring the phenology, distribution, and population density of pest species, and possibly for their control by mating disruption of adult elaterids (Reddy and Tangtrakulwanich 2014). Since the preliminary and incorrect work done on the *Limonius* spp. in the 1960s-70s, the technologies and methodologies for collection, identification, synthesis, and bioassays of volatile insect pheromones have dramatically improved, allowing for faster pheromone identifications from smaller amounts of material than were previously possible. Pheromone-based monitoring has great potential to help with the assessment of elaterid pest presence and identity, and conversely in

conservation and biodiversity estimates of endangered or rare click beetle species (Tolasch et al. 2007; Svensson et al. 2012; Tolasch et al. 2013; König et al. 2016).

The primary goal of this dissertation research was to build a foundation for click beetle pheromone research in the United States, by developing protocols for identifying and field testing these pheromones efficiently. The specific objectives were: (1) to identify the sex attractants and/or female-produced pheromones used by a number of North American click beetle species; (2) verify and optimize the pheromones in field bioassays.

Chapters 2 and 3 focus on the chemical ecology of two species in the genus *Cardiophorus* Eschscholtz. Specifically, Chapter 2 describes the identification and field bioassays of a sex attractant and female-produced sex pheromone for *Cardiophorus tenebrosus* LeConte and *C. edwardsi* Horn. This pheromone identification stemmed from the serendipitous discovery that males of both species were attracted to a known longhorned beetle (Coleoptera: Cerambycidae) pheromone. Both bioactive compounds contained chiral centers, and electrophysiological and field bioassays were used to determine their chirality and whether or not the presence of the other enantiomer had an effect on attraction. The findings in chapter 2 also constitute the first successful pheromone identification for any North American click beetle species.

Chapter 3 is a follow-up investigation on the specificity of the responses of *Cardiophorus* males to three compounds that were structurally similar to that of the female-produced pheromone and the sex attractant reported in chapter 2. In this study, only one of the pheromone analogs was attractive, which suggests that chirality, chain

length, and three-dimensional spatial relationships are all important factors for the recognition of pheromonal ligands by pheromone receptors of these species. In addition, the bioactive pheromone analog also had unprecedented levels of hyperactivity for *C. edwardsi* when compared to the actual pheromone, attracting more than 25 times as many males as equivalent doses of the actual pheromone in field trials.

Chapter 4 reports on the identification of a sex pheromone for the corn wireworm, *Melanotus communius* (Gyllenhal), which is a major pest of multiple crops in the eastern United States. Two pheromone candidates were identified from females. However, electrophysiological and field bioassays determined that only the major component elicited responses from males. Additional field bioassays determined the optimal dose of the active pheromone component and also compared total captures of male beetles using two different types of release devices. The results from this study should help with the commercialization of a lure that will hopefully be a new tool for management of this pestiferous species.

Lastly, chapter 5 describes the identification of the female-produced sex pheromone of *Idolus californicus* (Schaeffer). Field testing two blends of known elaterid pheromones revealed that *I. californicus* males were attracted to a neryl ester blend. Electrophysiological bioassays determined that two of the attractive blend components elicited responses from antennae of males, but field bioassays determined that only one component was attractive. Subsequent analyses of extracts of volatiles from female beetles showed that the females produced two compounds, one of which was the sex attractant identified in previous bioassays. In field bioassays with the pheromone

candidates, males were only attracted to traps that contained the major component, either as a single compound or in blends with the minor component. Males of a *Dalopius* species were also captured in traps that contained the minor pheromone component of *I. californicus*. Electrophysiological assays showed that the antennae of *Dalopius* males only responded to the minor component. The results from this study are the first to demonstrate conservation of pheromone structures between European and North American elaterids.

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Chapter 2: Identification of sex pheromones and sex pheromone mimics for two North American click beetle species (Coleoptera: Elateridae) in the genus *Cardiophorus* Esch.

Abstract

To date, all known or suspected pheromones of click beetles (Coleoptera: Elateridae) have been identified solely from species native to Europe and Asia; reports of identifications from North American species dating from the 1970s have since proven to be incorrect. While conducting bioassays of pheromones of a longhorned beetle (Coleoptera: Cerambycidae), we serendipitously discovered that males of *Cardiophorus tenebrosus* L. and *Cardiophorus edwardsi* Horn were specifically attracted to the cerambycid pheromone fuscumol acetate, (*E*)-6,10-dimethylundeca-5,9-dien-2-yl acetate, suggesting that this compound might also be a sex pheromone for the two *Cardiophorus* species. Further field bioassays and electrophysiological assays with the enantiomers of fuscumol acetate determined that males were specifically attracted by the (*R*)-enantiomer. However, subsequent analyses of extracts of volatiles from female *C. tenebrosus* and *C. edwardsi* showed that the females actually produced a different compound, which was identified as (3*R*,6*E*)-3,7,11-trimethyl-6,10-dodecadienoic acid methyl ester (methyl (3*R*,6*E*)-2,3-dihydrofarnesoate). In field trials, both the racemate and the (*R*)-enantiomer of the pheromone attracted similar numbers of male beetles, suggesting that the (*S*)-enantiomer was not interfering with responses to the insect-produced (*R*)-enantiomer. This report constitutes the first conclusive identification of sex pheromones for any North American click beetle species. Possible reasons for the strong and specific attraction of

males to fuscumol acetate, which is markedly different in structure to the actual pheromone, are discussed.

Introduction

Click beetles (Coleoptera: Elateridae) constitute a large family with ~10,000 described species, many of which are economically important (Barsics et al. 2013; Johnson 2002; Traugott et al. 2015). They are distributed worldwide and are some of the most abundant beetles in terrestrial habitats (Douglas 2011), including forests, grasslands, and croplands with organic matter in the soil. Elaterid larvae, called wireworms for some species, can inhabit the soil, plant litter, or dead and decaying wood (Johnson 2002; Traugott et al. 2015). Larvae can be phytophagous, predaceous, saprophagous, or omnivorous depending on species and availability of resources (Traugott et al. 2008; Vernon et al. 2009). Forest-inhabiting larvae can be saprophagous or predaceous on small invertebrates, and can be important natural enemies of many forest pests such as the pine looper, *Bupalus piniarius* (Dajoz 2000; Morris 1951; Turnock 1969; Zhang et al. 2008). Species which are agricultural pests are usually generalists, feeding on seedlings, roots, and harvested parts (e.g. tubers) of a variety of crops such as wheat, corn, and potatoes (Evans and Hogue 2006; Traugott et al. 2015).

The life histories of adult click beetles also differ with species and environmental variables. Most adults can fly and are able to disperse considerable distances (Schallhart et al. 2009). Species that feed as adults mainly consume rotting fruit, flowers, nectar, pollen, fungi, and sap from wounds, although some adult elaterids are predatory and will

feed on honeydew-producing hemipterans and the larvae of wood-boring insects (Evans and Hogue 2006; Johnson 2002). The majority of elaterid adults are nocturnal with activity periods correlated with temperature thresholds (Crozier et al. 2003; Edwards and Evans 1950; Evans and Hogue 2006). Some elaterid species mate only once (van Herk and Vernon 2014; Vernon and van Herk 2013), whereas others can mate multiple times (Furlan 1996).

To date, only a few pheromones and pheromone candidates have been identified from the estimated 10,000 species of Elateridae. Thus, pheromones or sex attractants have been identified from approximately 40 species native to Europe and Asia (König et al. 2015; Tolasch et al. 2013; Tóth 2013). All examples to date indicate that the family uses female-produced sex pheromones (König et al. 2015; Tolasch et al. 2013; Tóth 2013; Tóth et al. 2015). Practical applications of the pheromones for monitoring click beetles have been developed for species endemic to Europe and Asia (Tóth 2013). Ivashchenko and Adamenko (1980) showed that a paired ball-like structure in the abdomen of females of the species *Selatosomus latus* (Fabricus) is the likely source of pheromones. The first pheromones that were identified, from *Agriotes gurgistanus* (Faldermann), *A. litigiosus* Rossi, and *A. lineatus* L., were found in extracts of dissected pheromone glands (Yatsynin et al. 1980). Since then, a number of pheromones have been identified from *Agriotes* spp. (reviewed in Tóth 2013). Approximately two-thirds of the existing elaterid pheromone data are from this genus, most likely because many *Agriotes* spp. are important pests in Europe, and are of increasing importance as invasive species in North America (Furlan 2005; Furlan et al. 2001; Parker and Howard 2001; Vernon et

al. 2005). Sex pheromones have also been identified and developed to monitor rare or endangered species in Europe, such as *Elater ferrugineus* L. (Svensson et al. 2012; Tolasch et al. 2007; Zauli et al. 2014), and *Idolus picipennis* (Bach) (Tolasch et al. 2013). In Japan, pheromones are used for monitoring and management of species in the genus *Melanotus*, whose larvae are serious pests of sugarcane (Arakaki et al. 2008a, b, c; Iwanaga and Kawamura 2000; Kishita et al. 2003; Nagamine and Kinjo 1990).

To date, pheromones have not been identified from any of the estimated 965 North American elaterid species (Johnson 2002; Marske and Ivie 2003). Two putative pheromones, caproic and valeric acid, were reported for *Limonius californicus* (Mannerheim) and *L. canus* L. respectively (Butler et al. 1975; Jacobson et al. 1968), but field trials of these compounds in several US states failed to attract click beetles of any species (unpublished data: J. Serrano in California, P. Landolt and D. Horton in Washington state, G. Reddy in Montana), suggesting that these identifications were not correct. Although pheromones have not been identified, attraction of males to female beetles and/or extracts of females has been demonstrated in a few species, including *Agriotes ferrugineipennis* L. (Lilly and McGinnis 1965), *Ctenicera destructor* (Brown) (Doane 1961; Lilly and McGinnis 1965), *C. sylvatica* (Van Dyke) (Lilly and McGinnis 1965), *Hemicrepidius decoloratus* (Say) (Hayes and Wheeler 1968), *H. morio* L. (Chapman 1964), *L. californicus* (Jacobson et al. 1968; Lilly 1959; Lilly and McGinnis 1965; Lilly and McGinnis 1968; Shirck 1942;), and *L. canus* (Onsager et al. 1968). These data strongly suggest that females of these species are the pheromone-producing sex, in concordance with the data from European and Asian species.

While conducting field bioassays of longhorned beetle (Coleoptera: Cerambycidae) pheromones, we observed that male click beetles were sex-specifically attracted to traps baited with (*E*)-6,10-dimethylundeca-5,9-dien-2-yl acetate (fuscumol acetate). Fuscumol acetate is a known male-produced aggregation-sex pheromone for a number of species in the cerambycid subfamily Lamiinae (Hanks and Millar 2016). The click beetles were identified as *Cardiophorus tenebrosus* L. and *C. edwardsi* Horn (Fig. 1) based on the key by Blanchard (1889). *Cardiophorus tenebrosus* is reported to have a broad geographic range, encompassing much of the western United States, and with many morphological variations (Blanchard 1889). The *C. tenebrosus* that we collected matched the description of the California variety based on the structure of the male genitalia (Fig. 1) (Blanchard 1889). On the other hand, Blanchard (1889) reported that the range of *C. edwardsi* appears to be limited to California and Nevada, and it is not known to have any morphological or geographical variations.

Little is known about the biology of these two *Cardiophorus* species, especially *C. edwardsi*. Some notes on the basic biology and life history of *C. tenebrosus* were documented by Stone (1957). *Cardiophorus tenebrosus* has been reported from alfalfa and lima bean fields in Los Angeles and Ventura counties in California (Stone 1941; Stone 1957), in association with *L. californicus*. Based on these reports, it is unclear whether larvae of *C. tenebrosus* cause damage to alfalfa, but they do cause damage to lima beans (Stone 1957). No additional reports of damage caused by *C. tenebrosus* have been published, and their overall impact on crops remains unknown.

There is a clear need to expand on what little is known about elaterid pheromones, especially for North American species which are reemerging as important pests in some crops due to changes in pesticide use (Traugott et al. 2015; Vernon et al. 2009). Thus, the goal of this work was to follow up on the captures of *C. tenebrosus* and *C. edwardsi* in traps baited with fuscumol acetate. Our initial objectives were: (1) to determine which enantiomer of fuscumol acetate was most attractive, and whether the other enantiomer might interfere with attraction; (2) to analyze female-emitted volatiles to determine if females produce fuscumol acetate and/or other compounds. However, once it became clear that females did not produce fuscumol acetate, we added a third objective, that is, to identify, synthesize**,** and field test the true sex pheromones of these two species.

Methods and Materials

Insects and Collection of Volatile Compounds Adult beetles were collected from 9 June to 18 July 2016 and again from 21 April to 4 July 2017 at a site in the San Bernardino National Forest in San Bernardino Co., CA, USA, near Jenks Lake (34°09'45.8"N 116°54'08.6"W and 34°09′48.1″N 116°54′13.0″W). The sites are dominated by Ponderosa pine (*Pinus ponderosa* Douglas) and white fir (*Abies concolor* [Gordon]) (Pinales: Pinaceae), with some western black oak (*Quercus kelloggii* Newbury), canyon live oak (*Quercus chrysolepis* Liebm.) (Fagales: Fagaceae), big-cone Douglas-fir (*Pseudotsuga macrocarpa* [Vasey]) (Pinales: Pinaceae), and incense cedar (*Calocedrus decurrens* Torr.) (Pinales: Cupressaceae). Adult female *C. tenebrosus* were collected 21 April to 18 May 2017 from the ground around the periphery of other elaterid

pheromone traps, and *C. edwardsi* females were collected June 20-26 2017 from the ground and as random captures in flight intercept traps.

Headspace volatiles were collected from 1-4 live adult females for 1-7 d (Table 2.1) while they were held in 250 ml glass canning jars, with the metal lids fitted with an air inlet and outlet. Emitted compounds were trapped on a collector made from a glass tube (0.5 cm ID) with a 1-cm-long bed of activated charcoal (50-200 mesh; Fisher Scientific, Pittsburgh, PA, USA), held in place by glass wool plugs. Air was pulled through the jars at ~250 ml/min, and air entering the chamber was cleaned by passage through a copper pipe filled with granulated activated charcoal. The beetles were provided with a small metal screen to perch on, and sugar water for nutrition. Adsorbed compounds were eluted from the activated charcoal with 0.5 ml of CH_2Cl_2. The aeration extracts were then stored at -20 ºC until used in analyses.

It had been reported that the pheromone glands of female click beetles are located in the abdomen (Ivaschenko and Adamenko 1980; Merivee and Erm 1993), but we were unable to locate possible pheromone glands by dissections of females of our two study species. As a backup for *C. tenebrosus* females, following aerations, volatiles were collected from crushed abdomens of freeze-killed females, using solid phase microextraction (SPME). Thus, the abdomens of two *C. tenebrosus* females were placed in individual vials and crushed with a glass rod, the vials were capped with foil, and SPME fibers (100 μm PDMS; Supelco, Bellefonte, PA, USA) were inserted through the foil. Volatiles were collected onto the fibers for 2 h, then analyzed as described below.

Analyses of Extracts Extracts of volatiles were analyzed with an HP 6890 gas chromatograph (Hewlett-Packard, now Agilent, Santa Clara CA, USA) fitted with a DB-17 column (30 m × 0.25 mm ID × 0.25 μm film; J&W Scientific, Folsom CA, USA), coupled to an HP 5973 mass selective detector. The temperature program used was 100°C/1 min, then increased 10°C/min to 280°C. SPME fibers were desorbed in the injector port (250°C) in splitless mode for 30 sec prior to starting the run. Compounds were tentatively identified by mass spectral interpretation and matches with database spectra (NIST 98, Agilent), and then confirmed by matching retention times and mass spectra with those of authentic standards.

An aliquot of an extract containing the *C. tenebrosus* female-specific compound was catalytically reduced with H_2 and 5% Pd on carbon catalyst. After reduction, the catalyst was removed by filtration through a 5 mm bed of Celite in the tip of a Pasteur pipette plugged with glass wool. The reduced compound was then analyzed by GC-MS as described above.

The absolute configuration of the insect-produced methyl (6*E*)-2,3-dihydrofarnesoate was determined by base hydrolysis of the methyl ester, followed by formation of the diastereomeric amide with (*S*)-(–)-α-methylbenzylamine as described by Ho and Millar (2001b), with the exception that 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide hydrochloride (EDC) was used as the coupling reagent instead of dicyclohexylcarbodiimide (DCC) in the coupling step. Extracts of volatiles from *C. edwardsi* females were pooled to provide approximately 650 ng of methyl (6*E*)-2,3-dihydrofarnesoate. Samples of authentic racemic methyl (6*E*)-2,3-

dihydrofarnesoate and the (3*R*)-enantiomer were similarly derivatized to provide standards. The products were analyzed by GC and GC-MS on DB-5 and DB5-MS columns (20 m x 0.32 or 0.2 mm ID, respectively, J&W Scientific, Folsom CA, USA) in splitless mode. GC and GC-MS conditions were: 150ºC/0 min, 4ºC/min to 275ºC, injector temp 250ºC.

Gas Chromatography-Electroantennogram Detection (GC-EAD) GC-EAD analyses were conducted on an HP 5890 Series II GC fitted with a DB-17 column (30 m × 0.25 mm ID × 0.25 μm film). A glass X-cross split the effluent between the flame-ionization and EAD detectors, with helium being added through the fourth arm of the cross at 3 ml/min as makeup gas. The column effluent directed to the EAD was diluted in a humidified air stream (650 ml/min) and then into a glass tube (15 mm ID) that was directed over the antennal preparation. The antennal preparation consisted of an antenna from a male beetle, which was removed by pulling it off at the scape. The distal tip of the antenna was cut off with a razor blade, and then the antenna was placed between two saline-filled (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, and 0.20 g $NaHCO_3$ in 1 liter Milli-Q purified water) glass capillary electrodes. The electrodes were fitted with 0.2 mm diam gold wires that connected to a custom-built EAD amplifier. The signals from the GC and the amplifier were recorded simultaneously using Peak-Simple software (SRI International, Menlo Park CA, USA). The temperature program used for analyses was 100ºC/1 min, then 10ºC/minute to 275ºC, with an injector temperature of 250ºC. Extracts of volatiles were analyzed in splitless mode, and synthetic standards were analyzed in

split mode. SPME fibers were thermally desorbed directly into the GC injector as described above.

GC-EAD was also used to test the responses of the antennae of males of *C. edwardsi* to the enantiomers of fuscumol acetate, by alternating injections of equal amounts of each enantiomer at 3 min intervals, using an isothermal oven temperature (175ºC) in split mode, so that a single antenna was alternately exposed to each synthetic enantiomer up to five times within a given run.

Chemicals Racemic (5*E*)-6,10-dimethyl-5,9-undecadien-2-ol (fuscumol) and (*E*)-fuscumol acetate were purchased from Bedoukian Research (Danbury CT, USA). (*R*)- and (*S*)-fuscumol acetate were synthesized by enzymatic kinetic resolution of the racemic compound as described in Hughes et al. (2013). Racemic methyl (6*E*)-2,3-dihydrofarnesoate (henceforth "MDF") and methyl (3*R*,6*E*)-2,3-dihydrofarnesoate [henceforth "(*R*)-MDF"] were synthesized as previously described by Ho and Millar (2001a, b). A sample of methyl 3,7,11-trimethyldodecanoate was prepared by catalytic reduction of MDF, as described above.

Field Bioassays of Fuscumol Acetate The first field bioassays in which it was discovered that male *Cardiophorus* beetles were selectively attracted to fuscumol acetate were carried out at two sites approximately 500 m apart at the Jenks Lake site, from 12 May to 24 June 2016. Traps consisted of black cross-vane panel traps (AlphaScents, West Linn OR, USA) painted with Teflon emulsion (Fluon®, Bioquip Products Inc.,

Rancho Dominguez CA, USA) to render trap surfaces slippery, hung on 1.5 m tall, L-shaped stands made from PVC pipe. The treatments, which were meant to target cerambycids, consisted of a host plant volatiles blend (HPV) + ethanol as a positive control, and HPV and ethanol with racemic fuscumol, racemic fuscumol acetate, or racemic fuscumol + fuscumol acetate. The pheromones (1 ml of a 50 mg/ml solution in isopropanol) were dispensed from 5 × 7.5 cm (~0.05 mm thickness) low-density polyethylene zipper seal bags (Fisher Scientific, #01-816-1A). In addition, all traps were baited with a host plant volatiles (HPV) blend and ethanol. The HPV lure consisted of 10 ml of a synthetic conifer volatiles blend developed by Collignon et al. (2016), released from an open-topped 25 ml glass jar (4.3 cm tall × 4.3 cm outer diameter × 3.1 cm opening). Ethanol was released from 10 × 15 cm low-density polyethylene resealable bags (~0.05 mm wall thickness; Fisher Scientific) loaded with 50 ml ethanol. Traps were checked twice weekly and their order was rerandomized at every check. Pheromone lures were replaced weekly, the HPV blend was replenished twice per week, and ethanol was replenished as needed.

In a follow-up bioassay to determine which enantiomer of fuscumol was attractive, the treatments initially were isopropanol solutions of (*R*)-fuscumol acetate (25 mg/ml), (*S*)-fuscumol acetate (25 mg/ml), racemic fuscumol acetate (50 mg/ml), or isopropanol as the control. Lures contained 1 ml of the lure solution (in isopropanol) dispensed with the zipper seal bags as described above. This experiment was conducted from June 9-20, 2016 at the Jenks Lake site, using two spatial replicates that were separated by at least 300 m, and counted twice per week. After several trap checks, it was

apparent that (*S*)-fuscumol acetate was not attractive, and so this treatment was eliminated for the remainder of the season. The bioassay was continued from 20 June to 31 August, 2016 to determine whether there was any difference in attraction to racemic fuscumol acetate versus the (*R*)-enantiomer, i.e., whether the (*S*)-fuscumol acetate in the racemic material might interfere with attraction to the insect-produced (*R*)-enantiomer.

Field Bioassays of Methyl (6*E*)-2,3-Dihydrofarnesoate Modified intercept traps better suited to the ecological niches of adult elaterids were designed and deployed in field bioassays conducted in 2017 (Fig. 2.2). In particular, trap design and placement were adjusted because most adult elaterids are ground-dwelling or inhabit fallen decaying trees (Kabalak and Sert 2011), rather than flying higher in the canopy. Trap tops (30.5 cm × 30.5 cm) and vanes (30.5 cm × 10.2 cm) were made of corrugated plastic (McMaster-Carr, Santa Fe Springs CA, USA) and three vanes were used instead of the traditional four. Trap surfaces were again coated with Fluon® to improve trapping efficiency. Tops and vanes were held together by plastic zip ties. The bottom funnel (30 cm top diameter × 5.5 cm spout diameter × 12.1 cm height) was wired to the vanes, directing beetles into the collecting jar below. A ~8 cm hole was cut into the threaded lid of the collecting jar, and the funnel spout was hot-glued to the lid. Traps were not suspended above ground as in previous bioassays. Instead, the lower half of the trap was placed into a buried piece of PVC drain pipe (20.3 cm × 16 cm OD), up to the midpoint of the funnel.

For all experiments, traps were placed 10–15 m apart in transects, with treatments initially assigned randomly to traps. Traps were checked twice weekly, with lures

replaced weekly, at which time the trap order was rerandomized. Lures consisted of low-density polyethylene resealable baggies as described above, filled with 1 ml solutions of test compounds in isopropanol (doses listed below). Beetles were live trapped so that they could be brought back to the laboratory for sex determination, and for use in GC-EAD analyses.

The first bioassay of the likely sex pheromone of *C. tenebrosus* was deployed May 15-26 2017, with two spatial replicates. Treatments included racemic MDF (20 mg/ml) and fuscumol acetate (20 mg/ml), and an isopropanol control. On 26 May, the (*R*)-MDF treatment (4 mg/ml) was added to the field trial, the doses of racemic MDF and fuscumol acetate were correspondingly lowered to 8 mg/ml each, and bioassays were continued until 13 June. Doses were lowered because only small amounts of the (*R*)-enantiomer were available.

Field bioassays with *C. edwardsi* were conducted 9 June to 4 July 2017. Treatments consisted of racemic and (*R*)-MDF (8 and 4 mg respectively), racemic fuscumol acetate (8 mg), all in 1 ml of isopropanol in zipper seal baggies, and an isopropanol control. All experiments were conducted at the Jenks Lake site using traps and lures as described above.

Voucher specimens of both *Cardiophorus* species have been deposited in the Entomology Research Museum at UC Riverside with the following voucher numbers: *C. tenebrosus* males UCRC ENT 501449-501451; *C. tenebrosus* females UCRC ENT 184083-184087; *C. edwardsi* males UCRC ENT 502171-502172; and *C. edwardsi* females UCRC ENT 502991-502995.

Statistical Analyses Replicates for each field bioassay were based on both spatial and temporal replicates, with temporal replicates being the number of times the traps were checked. All statistical analyses were conducted with *R* version 3.4.2, used with RStudio version 1.1.383 (R-Development-Core-Team 2008; RStudioTeam 2015). Differences among treatment means were first assessed by the Kruskal-Wallis rank sum test, followed by Dunn's multiple comparison test using the FSA package (Dunn 1964; Ogle 2016).

Results

Identification of Fuscumol Acetate as a Sex Attractant for *C. tenebrosus* and *C. edwardsi* The first indication that fuscumol acetate might be a sex pheromone of *C. tenebrosus* and *C. edwardsi* was obtained during field trials of a number of cerambycid beetle pheromone components, when we noticed that large numbers of click beetles were caught only in traps baited with racemic fuscumol acetate. Further investigation determined that the trapped beetles were of two *Cardiophorus* species, and that all were males. In GC-EAD assays, antennae of males of both species responded strongly to racemic fuscumol acetate. Further GC-EAD analyses showed that the antennae of male *C. edwardsi* responded only to (*R*)-fuscumol acetate (Fig. 2.3). Analogous trials with antennae of male *C. tenebrosus* were not possible because the flight period of that species had ended, as evidenced by no further catches of that species in traps.

During the period June 9-20, 2016, a total of 227 *C. edwardsi* males were captured in traps baited with racemic and (*R*)-fuscumol acetate. Traps baited with (*S*)-

fuscumol acetate did not capture any beetles during this period. Because there were no antennal responses to (*S*)-fuscumol acetate and no beetles had been captured in traps baited with (*S*)-fuscumol acetate, a second experiment was carried out to compare the attraction of racemic versus (*R*)-fuscumol acetate, to determine if the presence of the (*S*)-enantiomer was antagonistic. Traps baited with (*R*)- and racemic fuscumol acetate captured totals of 513 and 212 *C. edwardsi* males, whereas solvent-baited controls caught no beetles at all. Although traps baited with (*R*)-fuscumol acetate captured more than twice as many beetles as traps baited with racemic fuscumol acetate, there was no statistically significant difference between the two treatment means (Kruskal-Wallis, $\chi^2_{(2)}=29.51$, $P<0.05$; Dunn's test, $P>0.05$) (Fig. 2.4). No female *Cardiophorus* were caught in any of the test treatments, indicating that attraction was sex-specific. In sum, these data suggested that (*R*)-fuscumol acetate was a possible sex pheromone of the two *Cardiophorus* species.

Identification of the Actual Sex Pheromone In 2017, females of both *C. tenebrosus* and *C. edwardsi* were collected from field sites. Analyses of extracts of volatiles collected from live females of both species revealed a small peak that elicited strong responses from antennae of conspecific male beetles in GC-EAD analyses (Fig. 2.5). The peak was detected in only one of five aerations of female *C. tenebrosus*, and in three of seven *C. edwardsi* aerations (Table 1). The retention times and mass spectra of the compound from females of both species were identical, suggesting that both species utilized the same compound as their pheromone. In addition, GC-EAD analyses showed that the aeration

extracts and SPME collections of volatiles from crushed female abdomens from two *C. tenebrosus* females contained the same single active component.

To our surprise, the retention time and mass spectrum of the active component in the extracts was quite different from those of fuscumol acetate (Fig. 2.6a-b), demonstrating that the actual pheromone could not be fuscumol acetate. The retention indices of fuscumol acetate and the active component on a DB-17 column were 1725 and 1874, respectively. The mass spectrum of the female-produced compound was characterized by a probable molecular ion at *m/z* 252 daltons, as evidenced by the loss of a methyl group to give a small ion at *m/z* 237 daltons, for a possible molecular formula of $C_{16}H_{28}O_2$, as compared to the molecular formula of fuscumol acetate of $C_{15}H_{26}O_2$. After catalytic hydrogenation, the reduced product displayed a small molecular ion at *m/z* 256 (Fig. 2.6c), indicating the presence of two C=C double bonds (or possibly one alkyne) in the parent compound. In addition, the mass spectrum and retention time of the reduced compound matched those of an authentic sample of methyl 3,7,11-trimethyldodecanoate, prepared by catalytic hydrogenation of methyl (6*E*)-2,3-dihydrofarnesoate. This crucial finding established the carbon skeleton of the pheromone. The positions of the two double bonds, and the stereochemistry of the double bond at C6 were then confirmed by matching the retention time and mass spectrum of the insect-produced compound with an authentic standard of methyl (6*E*)-2,3-dihydrofarnesoate. Finally, the absolute configuration of the insect-produced MDF was determined by hydrolysis of the methyl ester to the free acid, then formation of the amide with (*S*)-(−)-α-methylbenzylamine. The chromatograms of the resulting amide diastereomer from a composite extract from

female *C. edwardsi*, and of the corresponding amides prepared from synthetic racemic and (*R*)-MDF, demonstrated that female *C. edwardsi* produced exclusively the (*R*)-enantiomer (Fig. 2.7).

We then tested the responses of antennae of males to MDF and fuscumol acetate in GC-EAD analyses. Remarkably, the antennal responses to the two compounds were essentially the same (Fig. 2.8).

Field bioassays were then carried out with both racemic fuscumol acetate and racemic MDF. In the first such assay, a total of 90 *C. tenebrosus* males were caught. Traps baited with MDF caught significantly more beetles (81 males) than traps baited with racemic fuscumol acetate (9 males) (Kruskal-Wallis, $\chi^2_{(2)}=13.20$, $P<0.05$; Dunn's test, $P<0.05$). No beetles were captured in the isopropanol-baited controls.

In the second bioassay, comparing racemic MDF, (*R*)-MDF, and racemic fuscumol acetate, 71 *C. tenebrosus* and 169 *C. edwardsi* males were caught in total. The two MDF treatments attracted more male *C. edwardsi* than the fuscumol acetate (Kruskal-Wallis, $\chi^2_{(3)}=19.20$, $P<0.05$; Dunn's test, $P<0.05$) , whereas all three treatments were statistically equivalent for *C. tenebrosus* males (Kruskal-Wallis, $\chi^2_{(3)}=8.19$, $P<0.05$; Dunn's test, $P>0.05$ (Fig. 2.9). Control traps caught only one *C. edwardsi* male and no *C. tenebrosus* males.

Discussion

Data from our preliminary field bioassays and GC-EAD analyses suggested that (*R*)-fuscumol acetate was a possible and even likely female-produced sex pheromone for *C.*

tenebrosus and *C. edwardsi*. However, subsequent analyses of volatiles emitted by females of both species showed that *C. tenebrosus* and *C. edwardsi* females do not produce (*R*)-fuscumol acetate, but instead, produce a compound with a markedly different structure. Thus, fuscumol acetate appears to act as an effective mimic of the actual pheromone, as evidenced by the strong attraction of male beetles to fuscumol acetate, and by the responses elicited from antennae of male beetles when challenged with fuscumol acetate. Further analyses unequivocally revealed that female *C. tenebrosus* and *C. edwardsi* produce (*R*)-MDF as their sex pheromone, as confirmed by both GC-EAD analyses and field bioassays. This compound had been previously identified as a male-produced sex pheromone component for two stink bug species in the genus *Chlorochroa* (Ho and Millar 2001b).

Furthermore, GC-EAD analyses of the enantiomers of both MDF and fuscumol showed that males of the two *Cardiophorus* species apparently only detect the (*R*)-enantiomer of each of these two compounds. This was corroborated by the bioassay data, in which the racemate or the (*R*)-enantiomer of each compound were equally attractive, with no apparent decrease in trap catches being attributable to the (*S*)-enantiomer in the racemate of either compound.

Careful comparison of the structures of (*R*)-fuscumol acetate and (*R*)-MDF revealed structural similarities which might contribute to the mimicking effect (Fig. 2.10). In particular, the spatial relationships between the methyl group on C2 and the carbonyl group of the acetate of (*R*)-fuscumol acetate, and the methyl on C3 and the carbonyl on C1 of (*R*)-MDF appear to be crucial, because inversion of the chiral center in

each molecule completely eliminated both the EAG and the behavioral activity, despite the fact that the terpenoid hydrocarbon chain remains unchanged. This suggests that the pheromone receptor neurons are highly specific for that particular three-dimensional methyl-carbonyl structural fragment in males of both species. It remains to be seen whether other molecules containing this particular structural fragment, but different hydrocarbon chains, will also act as pheromone mimics for these two species.

Prior to this study, all known elaterid sex pheromones had been identified from European and Asian species in the subfamilies Elaterinae and Melanotinae. Based on the structures of their pheromones, three tribes within the Elaterinae can be divided into two groups (König et al. 2015; Tolasch et al. 2007). In the tribe Elaterini, *Elater ferrugineus* and *Sericus* spp. produce esters of methyl-branched alcohols with even numbered acids (König et al. 2015; Tolasch et al. 2007), such as 7-methyloctyl octanoate, with the methyl branch on the penultimate carbon of the alcohol fragment. In contrast, in the tribe Agriotini, several species in the genus *Agriotes* produce esters of acyclic terpenoid alcohols (i.e. geraniol, nerol, and farnesol) coupled with even numbered carboxylic acids (König et al. 2015; Tóth 2013). Recent work from König et al. (2015) and Tolasch et al. (2013) has shown that species in the *Idolus* and *Betarmon* genera (tribe Pomachiliini) produce some of the same terpenoid esters as members of the Agriotini.

So far, pheromones have only been identified from a single genus, *Melanotus*, in the subfamily Melanotinae. Several species in this genus have been shown to produce esters of 12 and 14 carbon saturated and unsaturated alcohols. For example, three *Melanotus* species from Japan produce dodecyl acetate and related esters with terminally

conjugated alcohols [(*E*)-9,11-dodecadien-1-ol butyrate and hexanoate] (Tamaki et al. 1986; Tamaki et al. 1990; Yen and Chen 1998). Four European *Melanotus* species were reported to produce a variety of esters based on 14 carbon unsaturated alcohols, such as tetradecenyl butyrate (Tolasch et al. 2007; Yatsynin et al. 1996), but structure identifications were incomplete and to our knowledge, no field bioassays were ever performed to determine the actual active pheromone components.

Fuscumol acetate as a sex attractant, and MDF as a sex pheromone, do not fit exactly into any of the chemical groups discussed above. However, they are both terpenoid esters with similarities to those produced by species in the tribes Agriotini and Pomachiliini (subfamily Elaterinae). The genus *Cardiophorus* Esch. currently is placed in the subfamily Cardiophorinae with no tribal designation (Douglas 2017), and does not belong to any of the elaterid taxonomic groups for which pheromones had previously been identified. In addition, these are the first confirmed identifications of pheromones for any North American click beetle species. Further research on the chemical ecology of other closely related species would help to determine whether members of this subfamily represent another distinct chemotaxonomic group of pheromone producers.

It is also interesting to speculate how *C. tenebrosus* and *C. edwardsi* remain reproductively isolated, given that they are at least partially sympatric, and appear to share exactly the same sex pheromone. One such mechanism may be temporal isolation due to different seasonal activity cycles. In particular, during our field studies, although we occasionally caught both species simultaneously, it was clear that *C. tenebrosus* was dominant earlier in the season, and then disappeared as we continued to catch *C.*

edwardsi. It is also possible that the two species may have somewhat different diel activity cycles. Furthermore, at close range, it is likely that species-specific contact pheromones in the cuticular lipids of the two species provide a further barrier to heterospecific mating attempts.

In summary, we have presented data showing the serendipitous but highly effective mimicry of the sex pheromone of two click beetle species, *C. tenebrosus* and *C. edwardsi*. Further work then identified the actual pheromone of both species as methyl (3*R*,6*E*)-2,3-dihydrofarnesoate. We hope that this first successful identification of sex pheromones for North American click beetle species will stimulate further work on the chemical ecology of these insects, for which a number of species are reemerging as pests of numerous agricultural crops due to changes in pest management practices.

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Figures and Tables

Species	No. of Individuals	Group No.	Duration	Pheromone Detected
C. tenebrosus	1		5 d	No
C. tenebrosus	1		5 d	No
C. tenebrosus	1		1 d	No
C. tenebrosus	1		3 d	No
C. tenebrosus	3	1	1 d	Yes
C. edwardsi	1		7 d	No
C. edwardsi	1		7 d	No
C. edwardsi	4	1	3 d	Yes
C. edwardsi	3	2	3 d	No
C. edwardsi	3	3-1[a]	3 d	Yes
C. edwardsi	3	3-2[a]	3 d	Yes
C. edwardsi	3	3-3[a]	4 d	No

Table 2.1 Aerations of females of *Cardiophorus tenebrosus* and *C. edwardsi.*
[a]consecutive aerations of the same group of individuals

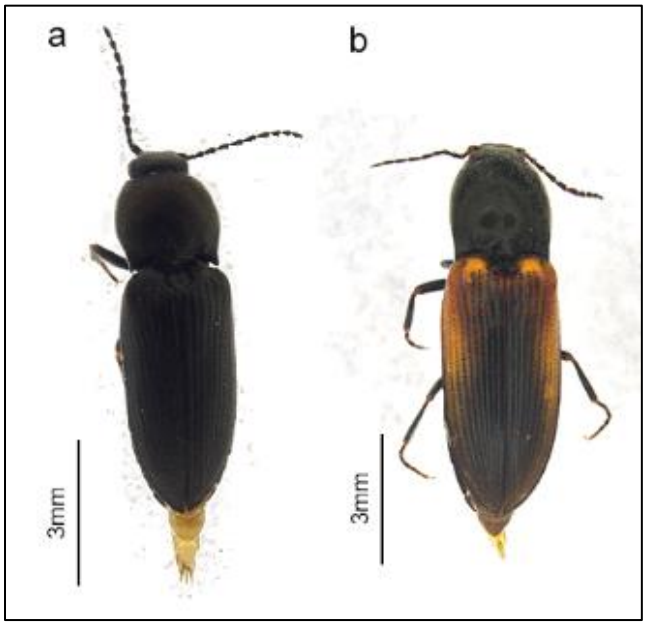

Figure 2.1 Dorsal images of *Cardiophorus tenebrosus* male **(a)** and *C. edwardsi* male **(b)**.

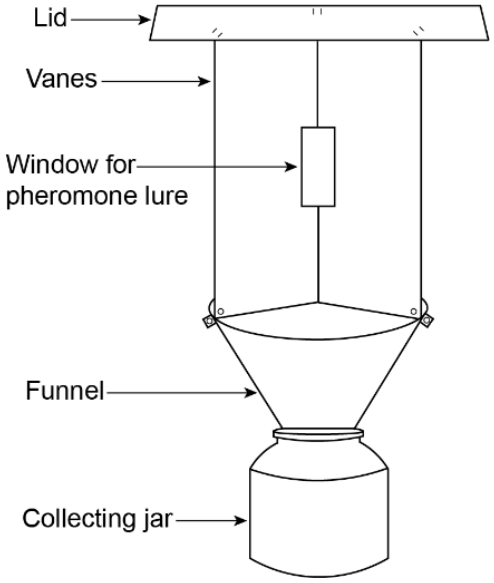

Figure 2.2 Schematic diagram of the modified intercept trap.

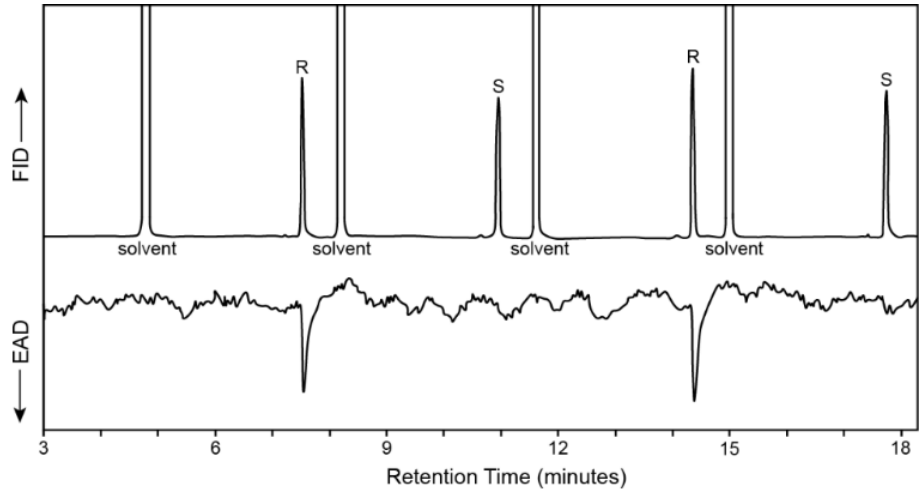

Figure 2.3 Representative GC-EAD analysis of alternating injections of (*R*)- and (*S*)-fuscumol acetate, stimulating an antenna from a male *C. edwardsi*. Top trace: GC chromatogram; bottom, inverted trace: antennal responses. Letters on tops of peaks indicate which enantiomer was injected; large peaks are from the solvent used to make sequential injections.

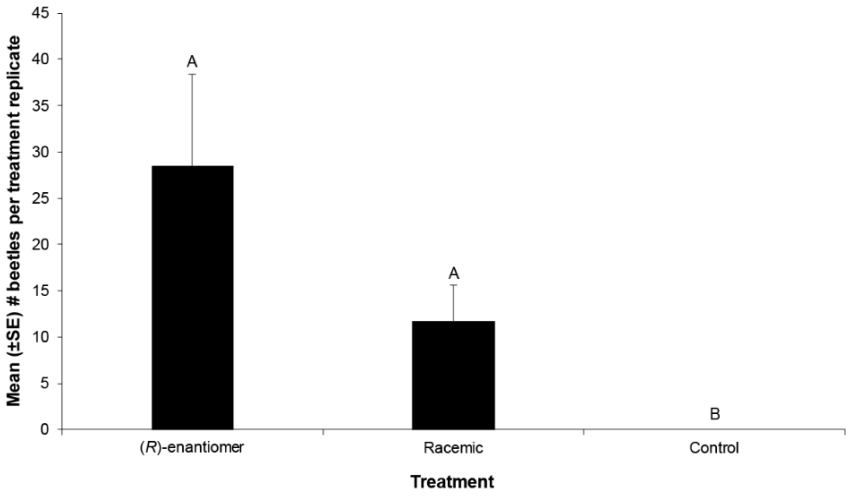

Figure 2.4 Mean (+SE) number of male *C. edwardsi* caught in traps baited with (*R*)- or racemic fuscumol acetate at the SBNF field site in 2016. The solvent control was isopropanol. Means with the same letter are not significantly different (Dunn's test, $P > 0.05$).

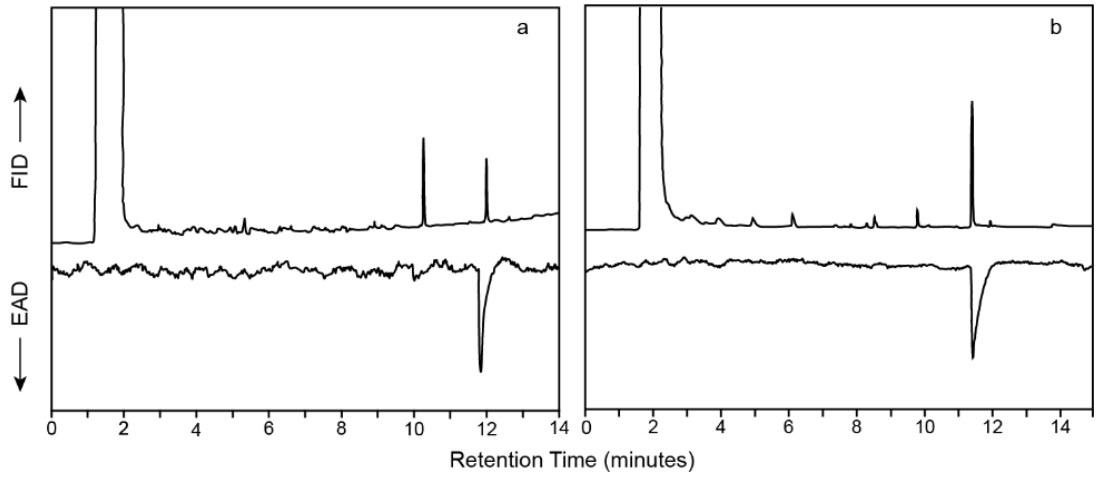

Figure 2.5 Representative GC-EAD analyses of extracts of odors from females of two *Cardiophorus* species. Top trace shows the GC chromatogram and the bottom, inverted trace shows the responses from antennae of conspecific males. **(a)** GC-EAD of *C. tenebrosus;* **(b)** GC-EAD of *C. edwardsi*.

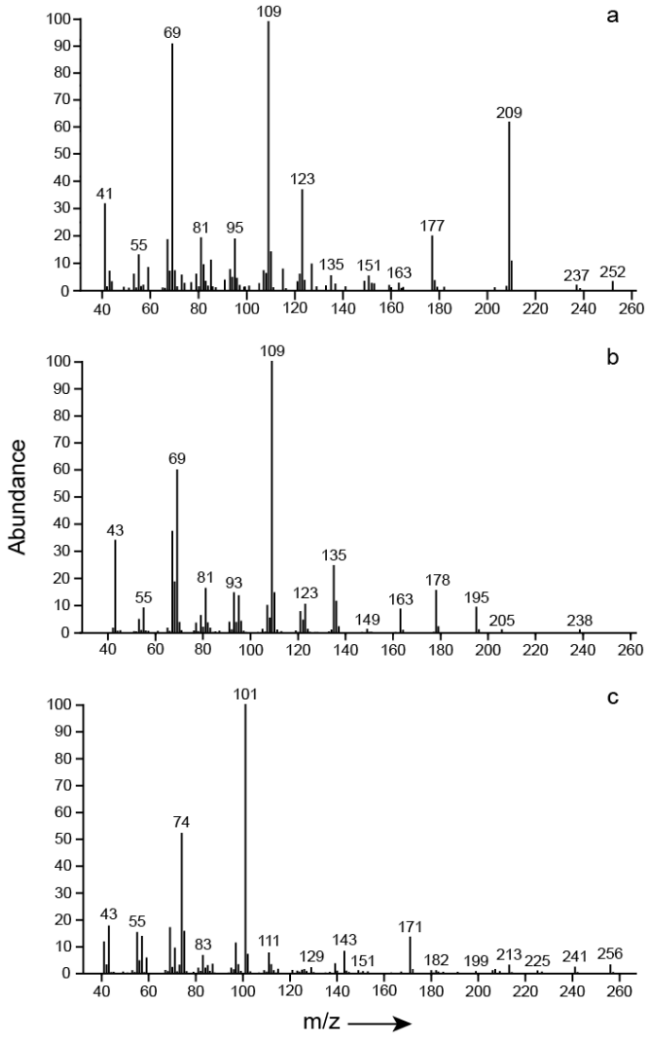

Figure 2.6 EI mass spectra (70 eV) of the compound emitted by female *C. tenebrosus* **(a)**; fuscumol acetate **(b)**; and the insect-produced compound after catalytic hydrogenation **(c)**.

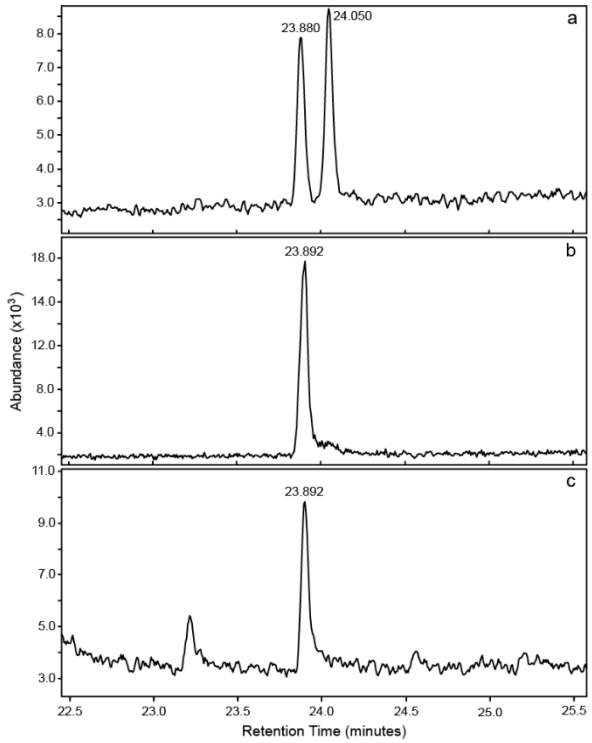

Figure 2.7 GC analyses of the (*S*)-(–)-α-methylbenzylamide derivatives of synthetic racemic MDF **(a)**, synthetic (*R*)-MDF **(b)**, and insect-produced MDF from *C. edwardsi* females **(c)**.

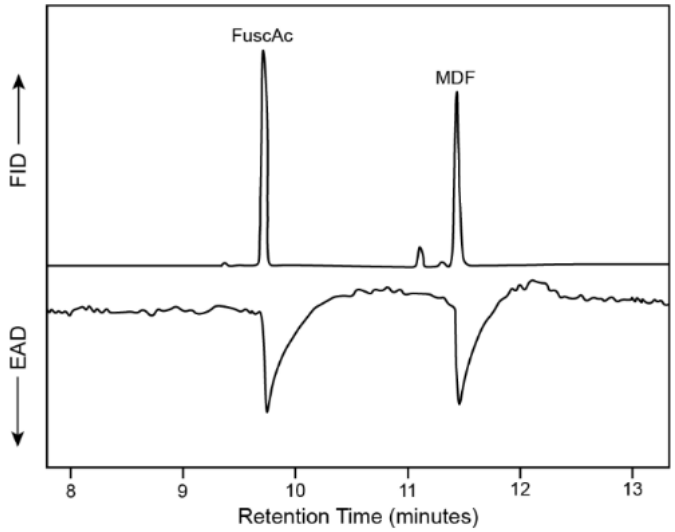

Figure 2.8 Representative GC-EAD analysis of synthetic fuscumol acetate (FuscAc) and MDF using an antenna from a male *C. tenebrosus*. Top trace shows the GC chromatogram and the bottom, inverted trace shows the antennal responses.

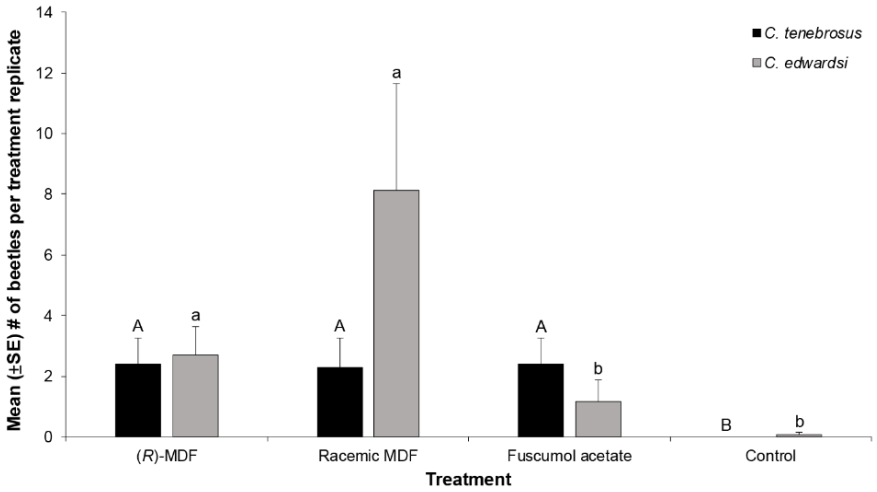

Figure 2.9 Mean (+1 SE) numbers of male beetles caught in traps baited with (*R*)-MDF, racemic MDF, or fuscumol acetate in isopropanol. The solvent control was isopropanol. Within each species, means with the same letter are not significantly different (Dunn's test, $P > 0.05$).

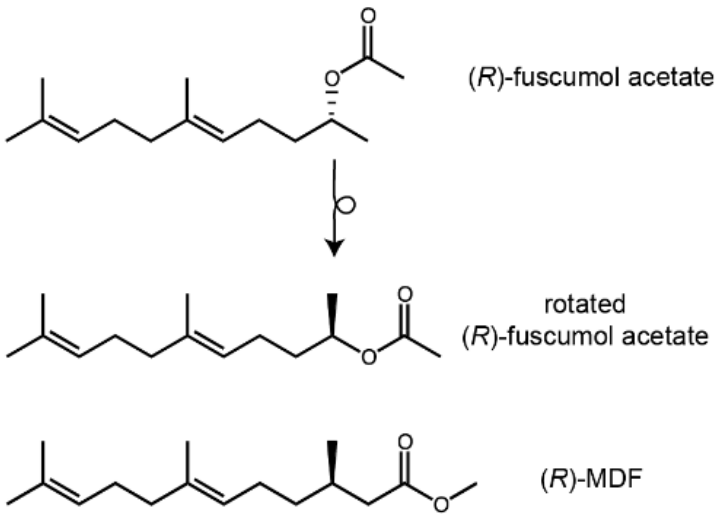

Figure 2.10 Comparison of the chemical structures of (*R*)-fuscumol acetate and (*R*)-MDF.

Chapter 3: Identification of a Hyperactive Pheromone Analog in Field Tests of Pheromone Mimics for Two Click Beetle Species in the Genus Cardiophorus (Coleoptera: Elateridae)

Abstract

Females of two click beetle species, *Cardiophorus tenebrosus* and *C. edwardsi* (Coleoptera: Elateridae), produce methyl (3*R*,6*E*)-2,3-dihydrofarnesoate as their sex pheromone. We had serendipitously discovered that males of both species were also strongly attracted to the (*R*)-fuscumol acetate ((*E*)-6,10-dimethylundeca-5,9-dien-2-yl acetate), a known longhorned beetle (Coleoptera: Cerambycidae) pheromone, due to its structural similarities to the actual pheromone. To further investigate the specificity of the responses of *Cardiophorus* males, additional analogs with different chain lengths and structural relationships compared to the natural pheromone were synthesized and tested. In field and electroantennogram bioassays, only fuscumol propionate ((*E*)-6,10-dimethylundeca-5,9-dien-2-yl propionate) elicited strong responses from *Cardiophorus* males, indicating that they were able to distinguish chain length and spatial relationships. In field trials, *C. tenebrosus* males were attracted equally to the analog and their natural pheromone, and both compounds elicited similar antennal responses from males. In contrast, traps baited with fuscumol propionate caught approximately 26 times as many *C. edwardsi* males compared to traps baited with the natural pheromone, although the analog elicited significantly smaller antennal responses from *C. edwardsi* males. Thus, in terms of behavioral responses, fuscumol propionate appears to be acting as a "hyperpheromone", a phenomenon which has rarely been observed in insect semiochemistry.

Introduction

Communication by means of volatile chemical signals is probably the most widespread form of intraspecific communication in the Insecta, and thousands of insect pheromones have now been identified from across the insect taxa. These communication systems are highly efficient, with behavioral responses being elicited by only a few molecules of pheromone. The signals are also usually species specific, with both the efficiency and the selectivity having been tuned by natural selection over millennia. During the last decades of the 20th century, a substantial amount of research was conducted on analogs of the attractant pheromones used by insect species, with a focus on the sex pheromones used in attracting a mate (reviewed by Renou and Guerrero 2000). These investigations had several different objectives. From a basic science viewpoint, structural modifications such as changes in the alkyl chain (elongation, shortening, saturation, and addition of alkyl branches), changes in the spatial and electronic structure by modification or replacement of polar groups (alcohols, esters, ketones, aldehydes), or isosteric replacements of hydrogen with fluorine were probed to see how they affected perception of and downstream behavioral responses to the analogs. Similar modifications were used to test whether the clearance of pheromones from receptors by enzymatic degradation could be disrupted. From a practical viewpoint, other types of pheromone analogs were developed as a possible means of replacing inherently unstable functional groups like aldehydes by more stable formate esters or methylene groups. However, almost without exception, it was found that at best, pheromone mimics were approximately equal in activity to the natural pheromones, or sometimes acted as synergists to the natural

pheromones. However, in most cases, the pheromone analogs varied from being much less attractive to not attractive at all compared to the natural pheromones, or they were actively inhibitory, disrupting responses to the natural pheromone when released in blends. Overall, this is perhaps not unexpected, given that pheromone receptors have evolved to be highly selective in order to recognize their particular ligands which are present in minute quantities in the sea of background odors present in natural environments. To our knowledge, the only instance in which any pheromone analog has been shown to exhibit activity greater than that of the natural pheromone (i.e., hyperpheromonal activity) was observed in the German cockroach *Blattella germanica*, whereby the three stereoisomers of (3*S*,11*S*)-3,11-dimethylnonacosan-2-one, a female-produced contact pheromone, elicited stronger behavioral responses from males at biologically relevant doses than the natural pheromone (Eliyahu et al. 2004).

While trying to identify the first pheromones for North American click beetle species (Coleoptera: Elateridae), we serendipitously discovered that a cerambycid beetle pheromone, (2*R*,5*E*)-6,10-dimethylundeca-5,9-dien-2-yl acetate (commonly known as (*R*)-fuscumol acetate) was a powerful mimic of the female-produced sex pheromone, methyl (3*R*,6*E*)-2,3-dihydrofarnesoate (henceforth "MDF"; structure shown in Fig. 1) of two click beetle species, *Cardiophorus tenebrosus* LeConte and *C. edwardsi* Horn (Serrano et al. 2018). The antennal responses in electroantennogram assays and the behavioral responses in field trials were so strong that we initially thought that fuscumol acetate was indeed the pheromone of these two species. Having finally identified the real pheromone from volatiles extracted from female beetles, and having compared the

beetles' responses to the two enantiomers of the mimic and the natural pheromone in both electroantennogram (EAG) and behavioral bioassays, we were struck by two points. First, in EAG bioassays, only the (*R*)-enantiomer of the pheromone or the mimic elicited any response at all from the antennae of male beetles, suggesting that the spatial relationship between the methyl group at the polar end of each molecule and the corresponding carbonyl group were crucial. Second, the all-or-nothing response elicited by that specific arrangement, despite the long hydrophobic chains of all four molecules being identical, suggested that the functionalized end of the molecule was most important, and that variation in the length of the hydrophobic end of the molecule might still result in active analogs, as long as the crucial methyl group to carbonyl motif was still intact. Here, we report the results of testing several pheromone analogs that were designed to probe these possibilities, and specifically, the identification of a remarkably active "hyperpheromone" which attracted approximately 26 times more *C. edwardsi* males than an equivalent dose of the natural pheromone in field bioassays.

Materials and Methods

Test Compounds Four compounds were synthesized for testing (Fig. 3.1). These included the known pheromone (*R*)-MDF as a positive control and baseline for comparison. In addition, three pheromone analogs were prepared, including the terpenoid esters:

1. (*E*)-6,10-dimethylundeca-5,9-dien-2-yl propionate (henceforth fuscumol proprionate) in which the chain length had been extended by one carbon, on the polar end of the chain. This compound most closely resembles the pheromone, MDF;

2. methyl citronellate, a monoterpenoid analog of MDF that shares the same three-dimensional methyl-carbonyl motif as MDF and fuscumol propionate, but lacks the terminal isoprene unit;

3. citronellyl propionate, which lacks the terminal isoprene group, and in which the distance between the crucial methyl and carbonyl groups is increased.

Racemic mixtures of each of the test compounds were used in field bioassays (described below). Although Serrano et al. (2018) found that *C. tenebrosus* and *C. edwardsi* males only respond to (*R*)-enantiomers of fuscumol acetate and MDF, the presence of the (*S*)-enantiomer (as a racemic mixture) did not cause a decrease in attraction to the (*R*)-enantiomer in the field.

Racemic MDF was available from previous work (Ho and Millar 2001). Racemic citronellyl propionate was synthesized as follows. Propionyl chloride (4.72 ml, 55 mmol) was added dropwise to an ice-bath cooled solution of citronellol (5.6 g, 50 mmol Aldrich Chemical), pyridine (4.4 ml, 55 mmol) and dimethylaminopyridine catalyst (100 mg) in 100 ml methylene chloride. After the addition was complete, the ice-bath was removed and the mixture was stirred 4 h at room temp. Residual propionyl chloride was then destroyed by addition of ethanol and stirring overnight. The resulting mixture was extracted sequentially with water, 1M HCl, saturated $NaHCO_3$, and brine, then concentrated to an odorous oil, which was >95% pure by GC, and was used without

further purification. ^{1}H and ^{13}C NMR spectral data agreed with those reported in Richter et al. (2014). MS (*m/z*, %): 138 (37), 123 (70), 109 (35), 95 (94), 81 (100), 69 (87), 57 (74), 41 (62).

Racemic fuscumol propionate was prepared in analogous fashion, substituting racemic fuscumol (Bedoukian Research, Danville CT) for citronellol. The product was purified by Kugelrohr distillation (oven temp ~99ºC, 0.25 mm Hg), yielding a colorless oil which was >95% pure by GC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.18 – 5.03 (m, 2H), 4.89 (m, 1H), 2.30 (q, J = 7.6 Hz, 2H), 2.14 – 1.90 (m, 6H), 1.70 – 1.65 (m, 3H), 1.59 (d, J = 7.4 Hz, 6H), 1.55 – 1.43 (m, 2H), 1.21 (d, J = 6.3 Hz, 3H), 1.13 (t, J = 7.6 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.28, 135.85, 131.52, 124.42, 123.54, 70.61, 39.83, 36.16, 28.11, 26.81, 25.82, 24.05, 20.15, 17.82, 16.05, 9.38. MS (*m/z*, %): 209 (2), 178 (12), 163 (7), 135 (18), 123 (9), 109 (100), 93 (16), 81 (20), 69 (59), 57 (37), 41 (31).

Racemic methyl citronellate was prepared by following the carboxylic acid methylation protocol described in Sun et al. (2016). Methyl iodide (1.44 g, 10.1 mmol) and K_2CO_3 (1.06 g, 7.64 mmol) were added to a solution of citronellic acid (0.85 g, 5 mmol; Aldrich Chemical) in dry DMF. The reaction mixture was stirred at 22°C for 30 min, then quenched with saturated aqueous $NaHCO_3$ (30 ml). The resulting mixture was extracted with hexane (3 × 50 ml) and the combined organic phases were washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The crude product was purified by vacuum flash chromatography, eluting with 10% EtOAc in hexane to yield a colorless oil (0.78 g, 84%) which was >95% pure by GC. ^{1}H and ^{13}C NMR spectral data agreed with those reported in Whittaker and Dong (2015). MS (*m/z*, %): 184 (M+, 7), 152 (39),

141 (2), 137 (5), 129 (4), 123 (4), 119 (3), 110 (62), 101 (12), 95 (67), 87 (12), 82 (32), 74 (24), 69 (100), 59 (23), 55 (40), 41 (69).

Field Bioassays Field bioassays were carried out from 9 April to 30 June 2018 at the same two sites near Jenks Lake (34°09'45.8"N 116°54'08.6"W and 34°09′48.1″N 116°54′13.0″W) in the San Bernardino National Forest in San Bernardino Co., CA, USA as the original studies described in Serrano et al. (2018). Modified flight intercept traps (Serrano et al. 2018) were placed 10–15 m apart, with treatments initially assigned randomly to traps. One block of treatments was tested at each field site. Traps were checked twice weekly and beetles were live trapped so that they could be brought back to the laboratory for sex determination and coupled gas chromatography-electroantennogram (GC-EAD) analyses. The lures were replaced weekly, at which time traps were rotated one position. Lures consisted of 5 × 7.5 cm (~0.05 mm wall thickness) low-density polyethylene zipper seal bags (Fisher Scientific, #01–816-1A), filled with 1 ml of a 20 mg/ml solution of test compound in isopropanol. Treatments consisted of methyl citronellate, citronellyl propionate, fuscumol propionate, and MDF (each as racemic mixtures), with isopropanol as a solvent control.

Gas Chromatography-Electroantennogram Detection GC-EAD analyses were conducted as described in Serrano et al. (2018), with the exception that the GC was fitted with a DB-5 column (30 m × 0.25 mm ID × 0.25 μm film; J&W Scientific, Folsom CA,

USA). Antennae from 4 *C. tenebrosus* males and 3 *C. edwardsi* males were used, with each antennal preparation being used for 1-3 analyses.

Quantification of Release Rates The release rates of MDF and fuscumol propionate were measured by performing headspace collections from the polyethylene lures loaded with 20 mg each of the two test compounds in 1 ml isopropanol. Collections (N=10) were conducted at 31 °C in a temperature controlled room. Volatiles were collected from the lures using two-piece cylindrical glass chambers (28 cm × 8 cm ID) with O-ring-sealed center joints and Swagelok® unions (Solon OH, USA) on either end for making connections. The lures (as described above) were hung on wire stands centered within the chambers and oriented perpendicular to the inlet and outlet. Air was pulled through the chambers at ~1 L/min using house vacuum, with incoming air being filtered through activated charcoal (6–14 mesh; Fisher Scientific). Volatiles were collected on 200 mg of thermally-desorbed activated charcoal (GAC-2050 ground down to ~100 mesh; Charcoal House, Crawford NE, USA) held between glass wool plugs in a short piece of glass tubing (0.9 cm ID). Collections were conducted for 4 h, beginning on the day the lure was made and then sampling again on days 3, 5, and 7. After collections were completed, trapped volatiles were eluted from the charcoal with 2 ml of dichloromethane containing dodecyl acetate (0.5 mg/ml) as in internal standard.

To quantify the amount of MDF and fuscumol propionate being released from the polyethylene lures, authentic standards of racemic MDF and racemic fuscumol propionate were diluted in hexane in a range spanning 4 different concentrations (1

mg/ml, 0.2 mg/ml, 0.1 mg/ml, and 0.02 mg/ml). The dilutions were then mixed 1:1 with a solution of dodecyl acetate (1 mg/ml in hexane) as an internal standard, and analyzed to prepare a calibration curve. Solutions were analyzed with an Agilent 78020A GC coupled to an Agilent 5977E MSD fitted with a DB-5MS column. The oven temperature was programmed from 50 °C for 1 min, then increased at 10 °C/min to 280 °C, with an injector temperature of 280 °C. Injections (1 µl) were made in splitless mode and the amounts of recovered pheromone were determined from the calibration curve.

Statistical Analyses Statistical analyses were conducted with *R* version 3.4.2, used with RStudio version 1.1.383 (R -Development-Core-Team 2017; RStudioTeam 2016). Replicates for field bioassays of both *Cardiophorus* species were based on both spatial and temporal replicates, with temporal replicates being the number of times the traps were checked. Differences among treatment means were first assessed by the Kruskal-Wallis rank sum test, followed by Dunn's multiple comparison test (Dunn 1964) using the "FSA" package version 0.8.23 (Ogle et al. 2019). The antennal responses of both male *Cardiophorus* species to MDF and fuscumol propionate were analyzed by comparing mean area counts of the response curves. The average release rates of racemic MDF and fuscumol propionate were compared within each day of the analysis. Both release rate and antennal response data were analyzed using the Fisher-Pitman nonparametric permutation test (Berry et al. 2002) using the "coin" package version 1.3 (Hothorn et al. 2019).

Results

Field Bioassays In field bioassays of the pheromone analogs with *C. tenebrosus*, a total of 408 males were caught between 12 April to 29 May 2018. Only traps baited with MDF and fuscumol propionate were significantly attractive compared to controls (Fig. 3.2a) (Kruskal-Wallis, χ^2 (4) = 99.13, $P < 0.05$; Dunn's test, $P < 0.05$), and the mean number of beetles in traps baited with MDF and fuscumol propionate were not statistically different. For *C. edwardsi*, a total of 941 males were caught from 18 May to 26 June 2018. In contrast to *C. tenebrosus*, traps baited with fuscumol propionate were ~26 times more attractive than traps baited with MDF, and both compounds were more attractive than all other treatments (Fig. 3.2b) (Kruskal-Wallis, χ^2 (4) = 83.63, $P < 0.05$; Dunn's test, $P < 0.05$). For both *C. tenebrosus* and *C. edwardsi*, traps baited with methyl citronellate and citronellyl propionate captured < 10 beetles total, and those treatments were not statistically different from the control (Dunn's test, $P > 0.05$).

GC-EAD Analyses Coupled GC-EAD analyses showed that only MDF and fuscumol propionate elicited detectable responses from the antennae of male *C. tenebrosus* and *C. edwardsi* (Fig. 3.3). For *C. tenebrosus* males, the antennal responses to MDF and fuscumol propionate ($N = 10$) were not significantly different in intensity (Fig. 3.3a; Fisher-Pitman permutation test, $P > 0.05$). In contrast, antennal responses ($N = 8$) from the antennae of male *C. edwardsi* were significantly larger to MDF than to fuscumol propionate (Fig. 3.3b; Fisher-Pitman permutation test, $P < 0.05$).

Release Rates The mean amount of racemic MDF and fuscumol propionate released by the polyethylene bags in a 4 h period were not significantly different within each of the four days that were sampled (Fig. 3.4; Fisher-Pitman permutation test, $P > 0.05$). The highest release rates for both MDF and fuscumol propionate were on day 3, and the mean (±SE) amounts released were 412 ± 109 µg and 490 ± 153 µg, respectively, during the 4 h collection periods.

Discussion

Our initial study in which we identified MDF as the female-produced sex attractant pheromone of the two study species suggested that several features of the MDF structure might be crucial for activity, specifically the methyl group on the chiral carbon, the carbonyl, the spatial relationship between these two functionalities, and the terpenoid hydrocarbon chain (Serrano et al. 2018). The cerambycid beetle pheromone, fuscumol acetate, shared these functional properties with MDF, which provides a plausible explanation for the strong attraction of male *Cardiophorus* beetles to this compound. The study described here further probed the roles of those structural features. Of the three pheromone analogs tested, fuscumol propionate was the most bioactive in both GC-EAD analyses and field trials, and structurally most similar to MDF, with the only difference between the two structures being the transposition of the oxygen and the methylene group flanking the carbonyl. These data, together with the good biological activity of fuscumol acetate, reinforced the importance of structural similarities as the ester end of the structures.

However, the additional importance of the structural details of the other end of the chain were dramatically illustrated by the fact that methyl citronellate, which has exactly the same structure as MDF at the ester end of the chain but is missing the terminal isoprene group, was completely inactive in both electrophysiological and field bioassays, for both species. In particular, the lack of EAD activity was unexpected because in our previous study, the (*R*)-enantiomers of fuscumol acetate and MDF elicited strong EAD responses, whereas the (*S*)-enantiomers, with identical structures except for the configuration of the methyl group closest to the carbonyl, elicited no discernable responses from antennae. This had suggested that the isoprenoid end of the structure was less important for structural recognition by the receptors (or possibly by pheromone binding proteins that transport pheromone molecules to receptors), but this is clearly not the case: both the isoprenoid and the ester portions of the pheromone structure are crucial for recognition, and hence any downstream behavioral response. This was further reinforced by the complete lack of activity of the third analog, citronellyl propionate, which both lacked an isoprene unit, and in which the carbonyl to methyl group distance was altered.

For *C. tenebrosus*, there was a relatively good correlation between the electrophysiological and behavior responses to fuscumol propionate and MDF. That is, the two compounds elicited similar antennal responses, which was congruent with the statistically equivalent attraction of beetles to traps baited with either compound. In contrast, no such correlation was found between EAD responses and trap catches with *C. edwardsi*: although fuscumol propionate elicited significantly smaller antennal responses

from antennae of males than MDF, traps baited with fuscumol propionate caught >25 times as many beetles as traps baited with an equivalent dose of the natural pheromone, MDF. This greatly amplified level of attraction could not be due to differing release rates of the two test compounds because their measured release rates were similar (Fig. 4).

To our knowledge, this level of agonistic "hyperactivity" of an insect-produced sex attractant pheromone is unprecedented. Among the many studies of pheromone analogs, if analogs or mimics have had appreciable activity at all, their activity typically has been less than or approximately equal in activity compared to the natural pheromone (reviewed in Renou and Guerrero 2000). In one rare exception, in which a pheromone analog appeared to be more attractive than the natural pheromone, trap catches of the gypsy moth, *Lymantria dispar*, in traps baited with 0.5 µg of an oxaspiropentane compound, 4-(1-oxaspiro[2.2]pent-2-yl)butan-1-ol, caught ~1.7 times as many moths as traps baited with 0.5 µg of the pheromone (+)-disparlure (Solari et al. 2007). However, those experiments may have been flawed, for several reasons. First, the estimated boiling points of 4-(1-oxaspiro[2.2]pent-2-yl)butan-1-ol and (+)-disparlure differ by ~100 ºC (244 vs. 341 ºC respectively; Advanced Chemistry Development Labs 2019), and consequently the vapor pressures of these two compounds differ by at least an order of magnitude (estimated with EPI Suite software; US EPA 2019). Consequently, the release rate of the analog in the field tests would have been substantially higher than that of (+)-disparlure, so estimates of the comparative attractiveness of the two compounds may have been confounded by the large differences in release rates, even though the dose of the analog used was one-tenth the dose of (+)-disparlure. In addition, crucial details about

the experiments were not given. For example, the trap dimensions were not explicitly stated, and the efficiency of sticky traps diminishes rapidly as they become saturated with with male moths, particularly relatively large moths such as gypsy moths. In addition, the traps in this study were spaced at ~60 m interval, i.e., far enough apart to create at least the potential for sampling different population densities. Lastly, traps were deployed for only 2-3 h, which suggests that the high numbers of moths trapped could be due to high populations and not trapping efficiency. Any one of these problems or some combination of them may have confounded the results of that study.

The ~26-fold higher trap catch of *C. edwardsi* in traps baited with fuscumol propionate as compared to MDF may be the result of one or a number of different factors, because the units of measurement (trap catch) are the end result of a series of behavioral steps. These in turn are mediated by both the chemical properties of the test compounds (e.g. volatility) and the biochemistry of the sequence of events from capture of an odor molecule by the insect's antennae through to the triggering of a behavioral output resulting in upwind orientation towards a trap. Differing volatilities of the attractive test substrates was ruled out because the measured release rates of fuscumol propionate and MDF were not statistically different. In terms of the biochemistry, it also seems unlikely that the rates of adhesion of the compounds to the antennal cuticle or dissolution in the antennal lymph would be markedly different given the very similar structures and consequently lipophilicities of the two molecules. However, slight differences in structure could indeed be crucial in the next steps. That is, capture of the ligands by a pheromone binding protein for transport through the sensillar lymph and transfer to the pheromone

receptor would both be expected to be sensitive to structure, and this was demonstrated indirectly by the complete lack of an EAD signal from the other two analogs tested, or of the (*S*)-enantiomers of MDF and fuscumol acetate (Serrano et al. 2018). Thus, the relative affinities of the transport proteins and the pheromone receptors, and the kinetics of the capture and transfer of fuscumol propionate and MDF between the transport proteins and the receptors, would likely be different. Similarly, the clearance of the pheromone from the active site of the receptor by pheromone degrading enzymes could also be different. These effects in turn could alter the signal transmitted to higher brain centers, and the eventual output to motor neurons resulting in attraction to the odor source. We also cannot rule out the possibility that fuscumol propionate and MDF may actually be detected by different receptors. At this point, all we can say is that the hyperattraction to fuscumol propionate demonstrated by the enhanced trap captures may be a consequence of any one of these factors, or some combination of them.

In summary, our results suggest that fuscumol propionate may be the most powerful analog or mimic ever reported for a volatile insect pheromone. This finding is even more remarkable in light of the fact that insect pheromone systems have been tuned by natural selection for millennia, to provide powerful and highly selective signals. Thus, it was entirely unexpected, and difficult to explain, how an analog could be so much more attractive than the natural pheromone. Further studies incorporating tools such as single sensillum recording might provide further insight into the mechanisms underlying the hyperactive response.

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Figures

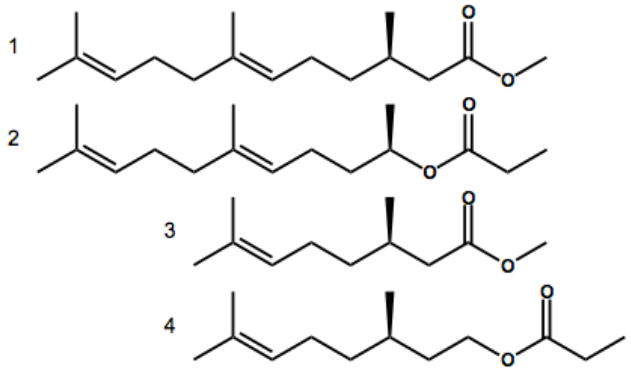

Figure 3.1 Structures of methyl (*R*)-dihydrofarnesoate (MDF) (**1**), (*R*)-fuscumol propionate when rotated at C2 (**2**), methyl (*R*)-citronellate (**3**), and (*R*)-citronellyl propionate (**4**).

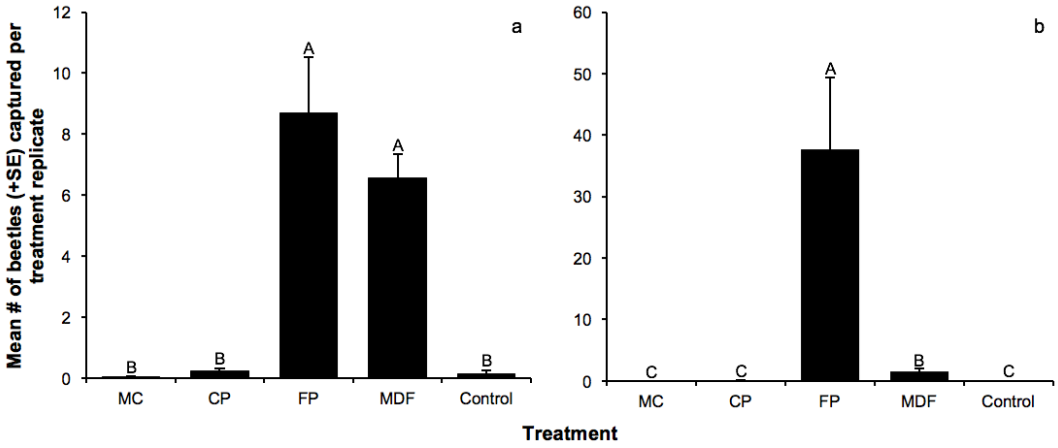

Figure 3.2 Mean (+1 SE) numbers of male beetles of (**a**) *C. tenebrosus* and (**b**) *C. edwardsi* caught in traps baited with racemic mixtures of MDF, fuscumol propionate (FP), citronellyl propionate (CP), and methyl citronellate (MC) in isopropanol. The solvent control was isopropanol. Within each species, means with the same letter are not significantly different (Dunn's test, $P > 0.05$).

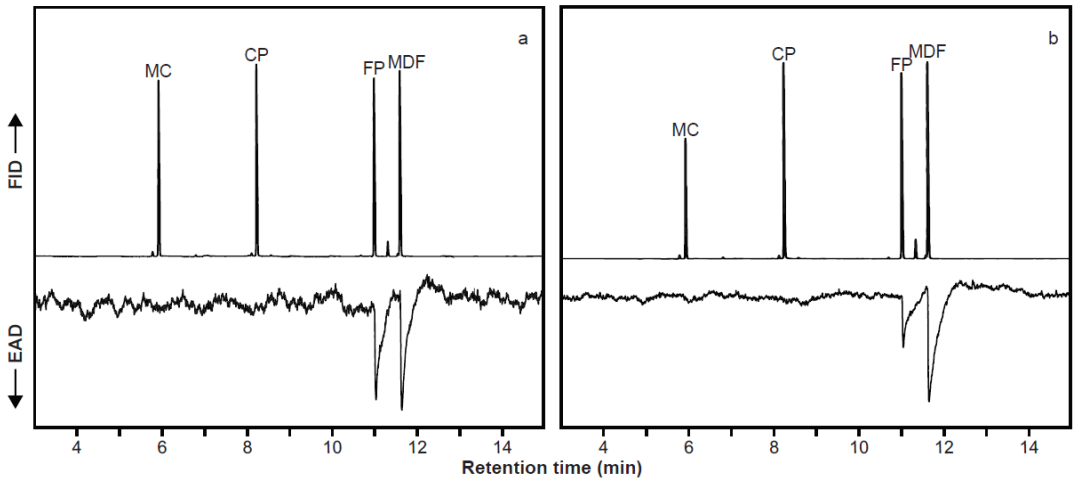

Figure 3.3 Representative coupled gas chromatography-electroantennogram detection (GC-EAD) analyses of racemic methyl citronellate (MC), citronellyl propionate (CP), fuscumol propionate (FP), and MDF using an antenna from a male *Cardiophorus* beetle. Top trace shows the GC chromatogram and the bottom, inverted trace shows the responses from antennae of *Cardiophorus* males. (**a**) GC-EAD of *C. tenebrosus*; (**b**) GC-EAD of *C. edwardsi*.

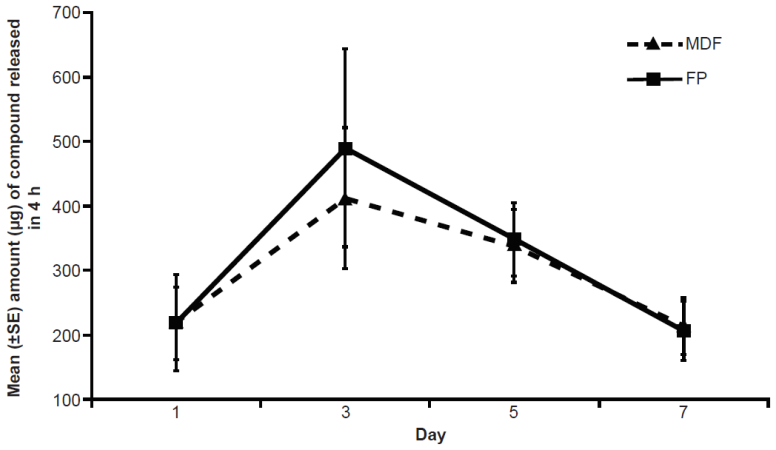

Figure 3.4 Mean (±1 SE) amounts (µg) of racemic MDF and fuscumol propionate released by the polyethylene bag lures in a 4 h period on days 1, 3, 5, and 7 after loading. Within each day sampled, there was no significant difference in release rates of the two compounds (Fisher-Pitman permutation test, $P > 0.05$, N = 10).

Chapter 4: 13-Tetradecenyl Acetate, a Female-Produced Sex Pheromone of *Melanotus communis* (Gyllenhal) (Coleoptera: Elateridae)

Abstract

Species-specific behavior-modifying chemicals have been used for more than 50 years for monitoring and management of insect pests of agriculture and human health. Insect pests that inhabit the soil have become increasingly problematic in recent years, in part due to the lack of effective management strategies. However, little is known about the insect-produced chemicals that mediate the reproductive behavior of these pests. We used chemical and behavioral studies to identify, synthesize, and field test the sex attractant pheromone of adults of *Melanotus communis*, commonly called the corn wireworm, the larvae of which are economically important pests of U.S. crops. Our results indicated that a single female-produced chemical was strongly attractive to conspecific male beetles, and did not appear to attract other species. In field evaluations, even a few milligrams of the lure attracted many males. In a trial comparing different slow-release dispensers, a small rubber septum impregnated with the chemical was as effective as and easier to use than a plastic bag dispenser. Given that the sex attractant of this insect consists of a single compound that can be readily synthesized, its development for monitoring and management of the corn wireworm may be economically feasible.

Introduction

The click beetles (Coleoptera: Elateridae) comprise a large and diverse family of insects, with >12,000 described species worldwide (P.J. Johnson, unpubl. catalog). Their larvae are known as wireworms, and can be significant agricultural pests. Their importance as

pests is increasing due to the phasing out of effective but environmentally detrimental insecticides that had been used in their control over the past several decades. Sex pheromones or sex attractants have been developed for monitoring and management of a number of Eurasian click beetle species. However, to date, pheromones have been identified from only two North American species, in the genus *Cardiophorus* Eschscholtz (Serrano et al. 2018), from the ~1,000 known species on this continent (Johnson 2002; Marske and Ivie 2003). Earlier reports of pheromone identifications for the North American species *Limonius californicus* (Mannerheim) (Jacobson et al. 1968) and *L. canus* LeConte (Onsager et al. 1968; Butler et al. 1975) have not been substantiated in subsequent field trials (unpublished data; JMS in California, P. Landolt and D. Horton in Washington state, G. Reddy in Montana).

In the eastern United States and southeastern Canada, the genus *Melanotus* Eschscholtz includes several economically important species, including *M. communis* (Gyllenhal), *M. depressus* (Melsheimer), and *M. verberans* (LeConte). In particular, *M. communis* is an important pest of maize, small grains, sugarcane, and vegetable crops in much of the United States east of the Rocky Mountains (Riley and Keaster 1979; Hall 1988, 1990; Jansson and Seal 1994; Lefko et al. 1998). Unfortunately, effective insecticidal options for in-season control of wireworms are disappearing, with currently registered insecticides being of limited efficacy (Kuhar et al. 2003; Larsen et al. 2016). There is some promise with sugarcane genotype resistance (Larsen et al. 2013) and some non-lethal methods could help to manage wireworms in sugarcane (Villani and Gould 1985a, b; Cherry and Nuessly 2010; Cherry et al. 2017). However, even low populations

of *M. communis* are a serious concern to sugarcane growers because it has been reported that just one wireworm per 1.5 m of sugarcane row can cause a reduction in stand and yield by 7.0% and 3.8%, respectively (Hall 1990). There is also a low market tolerance for injury to tuber/root vegetables, such as potatoes and sweet potatoes, due to the feeding holes caused by wireworms. These feeding holes can also facilitate attack by other invertebrates as well as pathogens. Thus, additional methods of monitoring and managing *M. communis* would be an asset for multiple cropping systems. One such method that has not yet been explored is the development of practical applications for sex pheromones of this species.

Pheromone glands of female *Melanotus fusciceps* (Gyllenhal) and *M. castanipes* (Paykull) from western Russia contained complex mixtures that included esters of 14-carbon saturated and unsaturated alcohols, but the exact structures and their evaluation in field trials have not been reported (Yatsynin et al. 1996). In contrast, research groups in Japan identified and field-tested female-produced sex pheromones for *M. okinawensis* Ôhira (dodecyl acetate) (Tamaki et al. 1986; Tóth 2013)and *M. sakishimensis* Ôhira ((*E*)-9,11-dodecadienyl butyrate and (*E*)-9,11-dodecadienyl hexanoate) (Tamaki et al. 1990; Tóth 2013) (*E*)-9,11-Dodecadienyl butyrate and (*E*)-9,11-dodecadienyl hexanoate have also been identified in extracts from *M. tamsuyensis* Bates, but field trials with the two compounds were not reported (Yen and Chen 1998). Practical applications for these pheromones have since been developed for monitoring purposes, and for control via mass trapping and mating disruption (Nagamine and Kinjo 1990; Kishita et al. 2003; Arakaki et al. 2008a, b, c).

The serendipitous discovery that male *Melanotus depressus* were attracted to (*E*)-11-tetradecenyl acetate and (*E*)-11-tetradecenol (Weires 1976; Brown and Keaster 1983), the pheromone components of the tufted apple bud moth (TABM), *Platynota idaeusalis* (Walker) (Hill et al. 1974), suggested that one or both of these compounds might also be pheromone components of *Melanotus* spp. However, to our knowledge, it was never determined whether *M. depressus* females actually produced (*E*)-11-tetradecenyl acetate and/or (*E*)-11-tetradecenol. This fragmentary data from several *Melanotus* spp. from North America and Eurasia suggested that the pheromones of species in this genus are likely to be esters of saturated or unsaturated 12- or 14-carbon alcohols.

Thus, the goal of the research reported here was to identify the sex pheromones of one or more North American *Melanotus* species, with the working hypothesis that the identification of the pheromone for one species would likely provide insight into the pheromones of congeners. As a first model species, we focused our attention on the economically important *M. communis*. Here, we report the identification, synthesis, and field-testing of 13-tetradecenyl acetate and several related compounds as possible female-produced sex pheromone components for this species.

Materials and Methods

Insect collection and rearing *Melanotus communis* adults were collected from agricultural fields at the USDA-ARS U.S. Vegetable Laboratory and Clemson University Coastal Research and Extension Center in Charleston, SC, USA, (32°44’44.06”N 80°03’40.35”W, elev. 4 m) intermittently from May to September in 2016 and 2017.

Beetles were collected with Malaise traps, black-light traps, at incandescent lights, and by hand under debris and vegetation in the fields. Malaise traps were used without a killing agent, and were monitored several times daily to collect live beetles. Light traps were monitored at night and again shortly after sunrise. Mating status, age, and other life history factors of the beetles were not known. After collection, adults were held individually in ventilated transparent 45 ml plastic vials (12 dram, No. 55-12, Thornton Plastics Co., Salt Lake City, UT, USA) streaked with honey and containing a piece of paper towel moistened with distilled water. Insects were held (22°C ± 1, 50% r.h., L:D 16:8) for 1 to 7 d prior to overnight shipment to the University of California, Riverside Entomology Quarantine Facility (USDA-APHIS permits P526P-14-03526 and P526P-17-02384). All *Melanotus* species were identified by comparison with reference specimens of known identity, supported with genital morphology and taxonomic keys (Quate and Thompson 1967). Voucher specimens are deposited in the Severin-McDaniel Insect Research Collection at South Dakota State University, the Entomology Research Museum at the University of California, Riverside, and the National Museum of Natural History through the USDA-ARS Systematic Entomology Laboratory, Washington, D.C.

Preparation and analysis of extracts of potential female-produced pheromones

Headspace volatiles were collected from individual live females held in 250 ml glass canning jars, with Teflon^{TM} lids fitted with an air inlet and outlet. The beetles were provided with 10% sugar water for nutrition (in a glass vial with a cotton-tipped applicator wick) and a small wire mesh perch. Emitted compounds were trapped on

collectors made from a glass tube (0.5 cm ID) with a 1-cm-long bed of activated charcoal (50–200 mesh; Fisher Scientific, Pittsburgh, PA, USA), held in place by glass wool plugs. For the female that was shipped to UC Riverside in June 2016, humidified and charcoal purified air was pushed through the glass jar at a flow rate of 250 ml/min for 4 d. In contrast, for collection of headspace volatiles from two females that were shipped to UC Riverside in June 2017, charcoal filtered air was pulled through the jars at 250 ml/min by applying a slight vacuum to the outlet. Headspace volatiles were collected for 3 d. All aerations were conducted in a temperature controlled room (23ºC), equipped with fluorescent lighting and a north-facing window (~5 m × 1 m) that provided ambient lighting. Trapped volatiles were eluted from the collectors with 0.5 ml of dichloromethane. After collections of headspace volatiles were terminated, volatiles were collected from individual crushed abdomens of freeze-killed females using solid phase microextraction (SPME) methods (Serrano et al. 2018).

Extracts of headspace volatiles were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) with an Agilent 7820A gas chromatograph coupled to an Agilent 5977E mass selective detector (Agilent Technologies, Santa Clara, CA, USA) fitted with an autosampler and an HP-5 column (30 m × 0.25 mm ID, Agilent). Injections were made in splitless mode, with the purge valve opened after 30 sec. The oven was programmed from 40°C for 1 min, then increased 10°C/min to 280°C. Volatiles from crushed abdomens that were adsorbed onto SPME fibers were analyzed with an Agilent 6890N gas chromatograph interfaced to an Agilent 5975C mass selective detector. The GC was also fitted with a HP-5 column and the temperature program used was 40°C for 1

min, then increased 10°C/min to 280°C, hold for 20 min. Loaded SPME fibers were desorbed in the injector port (250°C) in splitless mode for 30 sec prior to starting the run. Compounds were tentatively identified by mass spectral interpretation, matches with database spectra (W8N05ST; Wiley version 8.0 and NIST, version 5.0), and other published retention indices (Marques et al. 2000). Identifications were confirmed by matching retention times and mass spectra with those of authentic standards.

Coupled gas chromatography-electroantennogram detection (GC-EAD) was used to test the responses of the antennae of male *M. communis* beetles to various C14 acetates, specifically tetradecyl acetate, 13-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, and (*E*)-11,13-tetradecadienyl acetate. For all males, the legs were removed and genitalia were glued shut with Super Glue® before they were taken out of the quarantine facility (a requirement of the permit) for use in GC-EAD analyses. Analyses were conducted on an HP 5890 Series II GC (Hewlett-Packard, now Agilent) fitted with a DB-WAX column (30 m × 0.25 mm ID × 0.25 μm film; J&W Scientific). The effluent from the column was split using an 'X' cross, with half of the sample going to the flame-ionization detector and the other half to the antennal preparation, with the 4th arm of the X-cross providing make-up gas (~0.62 ml/min). The portion directed to the EAD was diluted in a humidified air stream (~550 ml/min) directed over the antennal mounting block (Serrano et al. 2018). Because the four acetates have similar retention times, GC-EAD analyses of the standards were done by alternating injections of the four compounds at ~2.5 min intervals using an isothermal oven temperature (220ºC) in split mode (injector temperature 250ºC), so that an antennal preparation was exposed sequentially to

each synthetic compound during a run (n=12 replicates, using one antenna each from four males).

Authentic standards of pheromone candidates Dodecyl acetate (henceforth 12:OAc) was purchased from TCI America (Portland, OR, USA), and 11-dodecenyl acetate (henceforth 11-12:OAc) and (*E*)-11-tetradecenyl acetate (henceforth *E*11-14:OAc) from Bedoukian Research (Danbury, CT, USA). Tufted apple bud moth sex pheromone lures were purchased from Scentry Biologicals Inc. (Billings, MT, USA).

Other compounds were prepared as described below. Unless otherwise specified, solutions were dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation under partial vacuum. Liquid chromatography purifications were carried out with 230-400 mesh silica gel. Tetrahydrofuran (THF) was purified by distillation from sodium benzophenone ketyl, and all reactions with air- or water-sensitive reagents were carried out in oven-dried glassware under argon.

Synthesis of tetradecyl acetate (henceforth 14:OAc) Tetradecyl acetate was prepared by dropwise addition of acetyl chloride (2.14 ml, 30 mmol) to an ice-bath cooled solution of tetradecanol (5.35 g, 25 mmol), pyridine (2.42 ml, 30 mmol), and dimethylaminopyridine (~100 mg) in 100 ml CH_2Cl_2. The mixture was then warmed to room temperature and stirred for 3 h, followed by addition of 1 ml EtOH and stirring for 1 h, to eliminate excess acetyl chloride as ethyl acetate. The mixture was then concentrated, and the residue was partitioned between hexane and water. The hexane

layer was washed sequentially with 1 M HCl, saturated aq. $NaHCO_3$, and brine, then dried and concentrated. The residue was purified by Kugelrohr distillation (oven temp ~105ºC; 0.15 mm Hg), yielding the ester as a clear, colorless liquid (5.60 g, 88%). ^1H NMR (400 MHz, $CDCl_3$): δ 4.03 (t, 2H, J = 6.8 Hz), 2.03 (s, 3H), 1.56 – 1.64 (m, 2H), 1.37 – 1.20 (m, 22H), 0.87 (t, 3H, J = 6.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.20, 64.64, 31.92, 29.65, 29.57, 29.52, 29.35, 29.26, 28.60, 25.91, 22.68, 20.98, 14.09. MS (*m/z,* %): 241 (M^+ -15, trace), 196 (3), 184 (trace), 168 (4), 154 (1), 153 (1), 140 (2), 139 (2), 130 (trace), 125 (7), 116 (4), 111 (19), 97 (39), 83 (52), 69 (51), 61 (41), 55 (53), 43 (100).

Synthesis of 13-tetradecenyl acetate (henceforth 13-14:OAc) A solution of 11-bromoundecanol (5.02 g, 20 mmol), dihydropyran (2.26 ml, 25 mmol), and ~50 mg p-toluenesulphonic acid (PTSA) in 50 ml ether was stirred overnight at room temperature. The mixture was then diluted with hexane, extracted twice with saturated aq. $NaHCO_3$ and once with brine, then dried and concentrated. The residue was purified by vacuum flash chromatography, eluting with 5% EtOAc in hexane, yielding 6.66 g of the THP-protected bromoalcohol (99%). This compound was taken up in 30 ml dry THF, and ~10 ml of the solution was added to a dry, argon-flushed flask charged with 0.72 g of freshly ground Mg chips. Once the Grignard reaction had started, as evidenced by warming of the flask and a grey color, the remainder of the solution was added to the flask over ~30 min. The mixture was then warmed to 50ºC and stirred for 1.5 h, then cooled to room temperature.

An oven-dried flask flushed with argon was charged with 300 mg CuI and 40 ml dry THF, and cooled to ~-10ºC in an ice-acetone bath. The Grignard solution was then added dropwise over 40 min with a syringe pump, producing a blue-black slurry. Allyl bromide (2.42 g, 20 mmol) in 5 ml THF was then added dropwise by syringe pump over 45 min, and the resulting mixture was stirred an additional 30 min, then quenched with saturated aqueous NH_4Cl, and diluted with hexane. The organic layer was washed with saturated aqueous NH_4Cl and brine, then dried and concentrated. The residue was taken up in 100 ml MeOH, ~50 mg of PTSA was added, and the mixture was stirred overnight at room temperature. Then, 5 g of solid $NaHCO_3$ were added, and most of the MeOH was removed by rotary evaporation. The residue was partitioned between hexane and water, and the hexane layer was washed with saturated aqueous $NaHCO_3$ and brine, then dried and concentrated. The crude product was purified by vacuum flash chromatography, eluting with 25% EtOAc in hexanes, followed by Kugelrohr distillation (oven temp ~80ºC, 0.05 mm Hg), yielding 3.33 g (15.7 mmol, 79%) of 13-tetradecenol. The alcohol was taken up in CH_2Cl_2, pyridine (1.58 g, 20 mmol) and dimethylaminopyridine (~100 mg) were added, and the solution was cooled in an ice-bath. Acetyl chloride (1.44 ml, 20 mmol) was added in one aliquot, and the mixture was warmed to room temperature and stirred for 3 h. The reaction was not complete, so a further 1 ml of pyridine and 0.5 ml of acetyl chloride were added, and the mixture was stirred an additional 2 h. EtOH (1.5 ml) was then added, and the mixture was stirred 1 h. The mixture was then concentrated, and the residue was partitioned between hexane and water. The hexane layer was washed sequentially with 1 M HCl, saturated aqueous $NaHCO_3$, and brine, then dried and

concentrated. The crude product was purified by Kugelrohr distillation, first removing a forerun fraction (oven temp <80ºC at 0.05 mm Hg, ~0.5 g), followed by distillation of the desired product (oven temp ~90ºC, 0.04 mm Hg), yielding 13-tetradecen-1-yl acetate (2.81 g, 70%), which gave a single peak on GC analysis. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.80 (m, 1H), 4.98 (br d, J = 14.1 Hz, 1H), 4.92 (br d, J = 10.2 Hz, 1H), 4.04 (t, J = 6.8 Hz, 2H), 2.07 – 1.98 (m, 5H), 1.64 – 1.56 (m, 2H), 1.43 – 1.22 (m, 18H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 171.19, 139.21, 114.08, 64.64, 33.81, 29.59, 29.55, 29.50, 29.25, 29.14, 28.94, 28.60, 25.91, 20.99. MS (*m/z,* %): 194 (M^+-AcOH, 5), 166 (3), 152 (2), 151 (2), 138(5), 137 (5), 123 (10), 109 (21), 105 (1), 96 (55), 91 (2), 82 (83), 77 (3), 68 (75), 61 (16), 55 (100), 51 (1), 43 (90).

Synthesis of (*E*)-11,13-tetradecadien-1-yl acetate (henceforth E11,13-14:OAc) A solution of THP-protected 11-dodecyn-1-ol (4.2 g, 16 mmol) and ~50 mg triphenylmethane indicator in dry THF was cooled to ~-10ºC in an ice-salt bath and stirred under argon. A solution of BuLi (8 ml, 20 mmol, 2.5 M in hexanes) was added dropwise, resulting in a bright red solution. Dry paraformaldehyde (4 g) was then added in one portion, and the mixture was warmed to room temperature overnight. The mixture was quenched with saturated aqueous NH_4Cl and extracted sequentially with hexane and ether. The combined organic layers were washed with water and brine, dried, and concentrated to a colorless oil which was added dropwise to a slurry of $LiAlH_4$ (1.32 g, 35 mmol) in 60 ml dry THF at 0ºC under argon. The mixture was heated to 65ºC for 2 h, then cooled in an ice-bath and quenched by dropwise sequential addition of 1.4 ml water,

1.05 ml 20% aqueous NaOH, and 4.9 ml water. The resulting slurry was stirred overnight, then filtered through a pad of Celite®, rinsing with ether. The filtrate was dried and concentrated, and the residue was purified by vacuum flash chromatography, eluting with 20% EtOAc in hexanes, yielding 2.75 g of the desired alkenol product as one peak by GC analysis. The purified alcohol was taken up in 150 ml CH_2Cl_2 and the solution was cooled in an ice-bath, then activated MnO_2 was added (29 g, 333 mmol), and the mixture was warmed to room temperature and stirred overnight. The mixture was filtered through a pad of Celite®, rinsing well with CH_2Cl_2. After concentration, the crude aldehyde was used directly in the next step.

A dry flask was charged with methyltriphenylphosphonium bromide (5.54 g, 15.5 mmol) and dry THF (100 ml). The slurry was cooled in an ice-bath, and potassium *t*-butoxide (1.68 g, 15 mmol) was added in one portion. The mixture was stirred at 0ºC for 15 min, then at room temperature for 3 h, yielding a milky yellow slurry. The slurry was cooled to 0ºC, and a solution of the crude aldehyde (1.9 g, ~6.4 mmol) in 10 ml THF was added by syringe pump over 1 h. The resulting mixture was slowly warmed to room temperature over 3 h, then quenched with saturated aqueous NH_4Cl. The mixture was extracted with hexane, and the hexane layer was washed with brine, dried, and concentrated. The residue was taken up in 50 ml hexane to precipitate most of the triphenylphosphine oxide byproduct, and the resulting slurry was filtered. The filtrate was concentrated, the residue was taken up in 25 ml MeOH, and ~50 mg PTSA was added. The solution was stirred for 3 h at room temperature, then 2 g solid $NaHCO_3$ were added, and the mixture was concentrated. The residue was partitioned between hexane and

water, and the hexane layer was washed sequentially with water and brine, then dried and concentrated and purified by vacuum flash chromatography, eluting with 25% EtOAc in hexane, yielding the dienol (1.03 g, 77%).

The dienol (0.53 g, 2.5 mmol) was acetylated as described above. The crude product was purified by Kugelrohr distillation (oven temp ~85ºC, 0.05 mm Hg), yielding (*E*)-11,13-tetradecadien-1-yl acetate as a colorless oil (0.52 g, 82%), 97% pure by GC. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.30 (ddd, J = 17.0, ~10.3 Hz, ~10.3 Hz, 1H), 6.04 (br dd, J = 15.2, 10.4 Hz, 1H), 5.70 (dt, J = 15.2, 6.9 Hz, 1H), 5.07 (br d, J ~ 17.5 Hz, 1H), 4.94 (br d, J ~ 10.2 Hz, 1H), 4.04 (t, J = 6.8 Hz, 2H), 2.10 – 2.02 (m, 2H), 2.04 (s, 3H), 1.64 – 1.58 (m, 2H), 1.42 – 1.24 (m, 14H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 171.24, 137.37, 135.59, 130.86, 114.57, 64.66, 32.55, 29.48, 29.46, 29.24, 29.18, 28.60, 25.90, 21.02. MS (*m/z*, %): 252 (M^+, 5), 209 (1), 192 (8), 177 (trace), 163 (4), 149 (7), 121 (23), 110 (16), 107 (16), 95 (39), 81 (71), 79 (71), 73 (3), 67 (100), 61 (9), 54 (53), 43 (92).

Field bioassays of synthetic pheromone candidates All field bioassays were conducted at the USDA-ARS U.S. Vegetable Laboratory and Clemson University Coastal Research and Extension Center in Charleston, SC. This shared facility encompasses a total of ca. 295 ha, of which ca. 95 ha are crop fields, with the remainder being buildings, wooded areas, and wetlands. In 2016, we tested pheromone candidates for *M. communis*, including the compounds identified from extracts of the insects, and several related compounds (Table 1). The main focus of this field trial was to determine the activity of the insect-produced 14:OAc and 13-14:OAc. In addition, the 12-carbon acetates were

included due to their structural similarities to the pheromone components of *M. okinawensis* (Tamaki et al. 1986). *E*11-14:OH, *E*11-14:OAc, and *E*11,13-14:OAc were tested to determine if they were attractive or synergized attraction to 13-14:OAc and 14:OAc, and *E*11,13-14:OAc was tested due to its structural similarities to pheromone components identified from *M. sakishimensis* and *M. tamsuyensis* (Tamaki et al. 1990; Yen and Chen 1998). The sex pheromone of the tufted apple bud moth (TABM), *Platynota idaeusalis,* (2:1 ratio of (*E*)11-14:OAc + (*E*)11-14:OH) was included because of reports (Weires 1976; Brown and Keaster 1983) of attraction of *Melanotus* spp. to TABM lures. Field trials were conducted for 6 wk (4 August – 13 September, 2016) at three fields; one planted to maize (0.52 ha), one to sweet potato (1.13 ha), and the other to cucumber (0.69 ha). Distance between these three fields ranged from 360 to 570 m. For all treatments (with the exception of the commercial TABM lure), ~10 mg of undiluted blend was pipetted into a 0.2 ml PCR tube (Multiply-Pro; Sarstedt AG & Co., Nümbrecht, Germany) with a pinhole in the lid made with a no. 3 insect pin. Empty, punctured PCR tubes were used as controls. Lures were suspended from the center of each trap. Lures were replaced and treatment locations in each transect were re-randomized biweekly. Linear trap transects were established at each field. Each transect consisted of seven Pherocon® 1C wing traps (Trécé Inc., Adair, OK, USA) deployed at the border between the field and an adjacent woodlot. Traps were spaced 12 m apart, and were hung 1 m above the ground from wooden stakes. Click beetles were removed from traps weekly and the adhesive was removed with xylene and an ultrasonic cleaner (Williams and O'Keefe 1990).

In 2017 field bioassays were conducted at 11 field sites for 18 wk (1 May – 6 September) with six test treatments and a control (Table 1). These fields were planted to maize, with the exception of two fields, one of which was planted to sweet potato, and the other was fallow. These 11 fields ranged in size from 0.32 to 0.77 ha, and were from 50 to 1210 m apart. The majority of the treatments were the same as the 2016 field trials, with the exceptions of the 12-carbon acetates and the quaternary mixture of 14-carbon acetates. *E*11-14:OAc and *E*11,13-14:OAc were added to a blend of 13-14:OAc and 14:OAc to determine if they synergized attraction to the pheromone candidates. Test blends were diluted with isopropanol (≥99.5% purity; Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 5.45 mg/ml. One milliliter of each treatment blend was pipetted into 5 × 7.5 cm (~0.05 mm thickness) low-density polyethylene zipper seal bags (#01-816-1A; Thermo-Fisher Scientific, Waltham, MA, USA), which were hung from the center of the traps. Vernon Pitfall Traps® constructed of black polypropylene were used, deployed in transects in the approximate center of each field, with traps in each transect spaced 12 m apart. These traps combine the attributes of pitfall and pheromone traps, and have been successfully used to test attractants for other click beetle species (van Herk et al. 2018). The killing agent in the traps was 12 ml of a 1:1 mixture of water and propylene glycol (Prestone LowTox® Antifreeze/Coolant; Prestone Products Corp., Lake Forest, IL, USA). Click beetles were removed from traps weekly and lures were replaced and treatment locations in each transect were re-randomized biweekly.

Dose-response test of 13-tetradecenyl acetate Having determined that 13-14:OAc was as good or better as an attractant than any blend of 14-carbon acetates, we conducted bioassays to determine the optimal dose of 13-14:OAc for attraction of *M. communis*. In 2018, a trial was set up for 8 wk (25 June – 20 August) in six fields; three planted to maize, and one field each to cucumber, tomato, and peanut. These six fields ranged in size from 0.38 to 0.73 ha, and were from 50 to 1135 m apart. At each field site a trap transect was established along the field border using five Vernon Pitfall Traps®, each 12 m apart. Test doses were 0.33, 1.0, 3.3, and 10 mg/ml of 13-14:OAc (1 ml loads), with an isopropanol control, dispensed from zipper-seal polyethylene bags as described above. Beetles were collected weekly, and lures were replaced and re-randomized every 2 wk.

Comparison of pheromone dispensers for 13-tetradecenyl acetate In 2018, a trial was set up for 7 wk (22 June – 10 August) to compare two lure devices at 12 fields different from those in the dose-response study described above. Eight of these fields were planted to maize, two fields to cucumber, and one field each to tomato and peanut. These 12 fields ranged in size from 0.38 to 0.73 ha, and were from 45 to 1779 m apart. At each field site, two Vernon Pitfall Traps® were deployed along the field border, 12 m apart. At each site, one of the traps was baited with a zipper-seal bag (as described above) and the other with a grey rubber septum (9 mm × 19 mm, 6.2 mm I.D.; West Co., Lionville, PA, USA; item # 1888 grey). The zipper seal bags were loaded with 1.0 ml of a 10 mg/ml solution of 13-14:OAc in isopropanol. Rubber septa were Soxhlet-extracted with dichloromethane for 8 h, then dried in a fume hood before being loaded with 0.2 ml of a

hexane solution of 13-14:OAc (50 mg/ml). Loaded septa were allowed to absorb the pheromone solution and dry, then were sealed in glass vials for shipment to South Carolina. Treatment locations were re-randomized and click beetles were collected weekly.

Diversity of *Melanotus* species at the field sites and cross-attraction. To gain a better understanding of the Melanotus species occurring at our field sites, two unbaited Malaise traps (Townes style; Sante Traps, Lexington, KY, USA) were set up at two of the fields (traps separated by 620 m) at the border between the woodlot and the field. These traps were deployed in April and discontinued in November 2016-2018. Collection vessels contained ca. 50 ml of a 1:1 mixture of water and propylene glycol as described above. Traps were serviced weekly. Melanotus specimens captured in these traps were identified to species as described above. Voucher specimens are deposited in the same museums noted above for the specimens collected for putative pheromone analysis.

Statistical Analysis Captures of *M. communis* were analyzed using a multi-response permutation procedure (MRPP) (Peck 2016). Data were square-root transformed prior to MRPP analysis using Euclidean distance to conduct the distance matrix (PC-ORD 7.0; MjM Software Design, Gleneden Beach, OR, USA) (McCune et al. 2002). Pairwise comparisons of treatments were Benjamini-Hochberg (Benjamini and Hochberg 1995) adjusted using a false discovery rate of 0.25. Unadjusted P and A (chance-corrected

within-group agreement) values are presented in the results. Capture data from 2018 were square-root transformed prior to t-test.

Results

Identification of pheromone candidates Coupled GC-MS analyses of extracts of headspace volatiles collected from live female *M. communis* did not show peaks from any likely pheromone candidates, but only traces of hydrocarbons and contaminants. However, GC-MS analyses of SPME-trapped headspace volatiles from three separate crushed abdomens of these same beetles revealed two major peaks in the total ion chromatograms (Fig. 4.1A) which were absent in the corresponding chromatograms of extracts of headspace volatiles from the live individuals. The two compounds were tentatively identified from matches of their mass spectra with database spectra as tetradecyl acetate and a monounsaturated analog (Fig. 4.1B). For the unsaturated 14-carbon acetate, the double bond was tentatively identified to be in the terminal position, by comparison of its Kováts retention index with the retention indices of all possible straight-chain monounsaturated 14-carbon acetates on a DB-5 column (Marques et al. 2000). The identifications of the two female-produced compounds were confirmed by matching their retention times and mass spectra with those of authentic standards.

Additionally, in GC-EAD analyses testing the synthesized pheromone components and analogs, antennae of male beetles were challenged sequentially with 14:OAc, 13-14:OAc, *E*11-14:OAc, and *E*11,13-14:OAc. Of the four compounds, 13-14:OAc consistently elicited the strongest responses from antennae of males (Fig. 4.2).

There were negligible antennal responses to *E*11-14:OAc, and smaller and inconsistent responses to 14:OAc and *E*11,13-14:OAc (Fig. 4.2).

Comparison of synthetic sex pheromone candidates In preliminary bioassays in 2016, a total of 26 male *M. communis* were caught, with lures containing 13-14:OAc being most attractive (Fig. 4.3). In particular, 13-14:OAc as a single component attracted marginally more beetles (MRPP; P = 0.053, A = 0.081) than the 4:1 binary blend of 13-14:OAc + 14:OAc, but not more ($P > 0.05$) than the 1:1 blend of these two compounds. Captures in the 1:1 and 4:1 blends of 13-14:OAc + 14:OAc were not significantly different (P > 0.05). The 4:1 blend of 13-14:OAc + 14:OAc did not differ (P > 0.05) from the control. No beetles were caught in the traps baited with 14:OAc, the 12:OAc + 11-12:OAc blend, or TABM pheromone.

In 2017, using plastic bag lures and Vernon Pitfall Traps® instead of plastic tube dispensers and sticky traps, 449 male *M. communis* were attracted to treatments containing the 14-carbon acetates, and trends were similar to those of 2016. The 4:1 and 1:1 blends of 13-14:OAc + 14:OAc and 13-14:OAc as a single component were not significantly different (Fig. 4), whereas all of these were significantly more attractive than the 1:1:1:1 blend of 13-14:OAc+14:OAc+(*E*)-11,13-14:OAc+(*E*)-11-14:OAc (Fig. 4.4). The TABM pheromone, 14:OAc, and control treatments attracted one, two, and five males, respectively, and did not differ from each other (P > 0.05).

Dose-response tests with 13-tetradecenyl acetate In the 2018 trial using a semi-log range of doses, a total of 135 male *M. communis* were captured. The 10 mg dose was significantly more attractive than the lower doses (Fig. 4.5; MRPP 10 vs 0.33; P = 0.002, A = 0.318) (MRPP 10 vs 1; P = 0.001, A = 0.401) (MRPP 10 vs 3.3; P = 0.018, A = 0.165). Captures with the 0.33, 1.0, and 3.3 mg doses were not significantly different from each other (P > 0.05), but were significantly greater than the control.

Comparison of pheromone dispensers for 13-tetradecenyl acetate In this trial comparing equal doses of pheromone released from plastic bag or rubber septum dispensers, a total of 149 male *M. communis* were captured (septa = 76; bags = 73). Captures in traps baited with the polyethylene bags declined in an exponential manner from 3.33 males per trap in wk 1 (22-29 June) to zero in wk 4 (13-20 July), after which trap catches were negligible (Fig. 4.6). Captures in traps baited with the rubber septa were relatively stable, i.e., ca. 1.6 males per trap per week for 4 wk (22 June-20 July), after which trap catches declined (Fig. 4.6). For both dispenser types, trap catches after wk 4 were at or near zero. Catches in traps baited with the plastic bags were not significantly different (P > 0.05) than those in traps baited with septa at wk 1 and 2 (22-29 June and 29 June – 6 July), but were significantly less than the septa in wk 3 (6-13 July; $T_{22} = 2.42$, P = 0.025) and 4 (13-20 July; $T_{10} = 3.78$, P < 0.0001).

Diversity of *Melanotus* species at the field sites and specificity of pheromone lures Of the 758 *Melanotus* individuals captured in traps baited with test chemicals during the

pheromone field bioassays, all but two were male *M. communis*. The exceptions were one male *M. lanei* Quate (1-8 May 2017) and one female *M. piceatus* Blatchley (8-15 May 2017), both of which were captured in traps baited with the 1:1 mixture of 13-14:OAc + 14:OAc. An additional 85 *Melanotus* individuals were captured in Malaise traps, 43 of which were *M. communis*. The remaining *Melanotus* captured in the Malaise traps were: *M. verberans* (15)*, M. morosus* Candèze (3)*, M. piceatus* (15), *M. americanus* (Herbst) (1), *M. corticinus* (Say) (5), and *M. similis* (3), but no males of any of these species were caught in any of the lure-baited traps, indicating a high species specificity of the *M. communis* pheromone.

Discussion

Our analytical and bioassay data indicate that 13-14:OAc is the major and likely only component of the female-produced sex pheromone of *M. communis*. Male *M. communis* were not attracted to lures that did not have 13-14:OAc as a component. The analog 14:OAc, although present in volatiles collected from crushed abdomens of females, appeared to be inactive, neither increasing or decreasing attraction of males. In addition, 14:OAc elicited minimal responses from antennae of males in GC-EAD bioassays, further suggesting that it is not a crucial component of the pheromone. Thus, 14:OAc may have a role as an inhibitory compound preventing cross-attraction of congeners that might also use 13-14:OAc as a pheromone component, or it may simply be present as a biosynthetic precursor to the pheromone or even an artifact, because our analyses were based on volatiles collected from crushed abdomens rather than the volatiles actually

released by calling females, because none of the females emitted detectable amounts of these or related compounds under laboratory conditions.

In tests of analogs of 13-14:OAc, the TABM pheromone blend was not attractive to *M. communis*, and *E*11-14:OAc elicited no responses from antennae of males in GC-EAD assays. In contrast, *E*11,13-14:OAc did elicit EAD responses, but if anything, the field bioassay data from 2017 suggested that it might be inhibitory (Fig. 4). Thus, the EAD responses seen may have been a result of the structural similarity between *E*11,13-14:OAc and the actual pheromone, 13-14:OAc.

In our initial trials in which test pheromone blends were deployed in plastic PCR tubes with a pinhole in the cap, only low numbers of beetles were caught, suggesting that the release rate of these dispensers might be too low. This was corroborated in subsequent trials in which either small plastic bags or rubber septum dispensers loaded with similar doses of pheromone attracted substantially more male beetles than the PCR tube dispensers. A dose-response bioassay testing a semi-log range of doses showed that the 10 mg dose, dispensed from plastic bags, was significantly more attractive than any of the lower doses. In a follow-up field trial, the plastic bag dispensers were compared to the rubber septa which have found widespread use as pheromone dispensers for lepidopteran species, with both dispensers loaded with 10 mg of pheromone. Under typical summer climatic conditions, the septa appeared to have longer and more consistent field lifetimes than the polyethylene bags. Specifically, the two dispenser types attracted similar numbers of beetles for the first two weeks after deployment, after which the attractiveness of the plastic bags appeared to decrease more quickly than that of the

rubber septa. Five weeks after deployment (i.e., late July), both lure types attracted minimal numbers of beetles, but this may have been due to the natural seasonal decline in *M. communis* reproductive activity.

During our field trials, conducted over several years with a number of potential pheromone candidates for *Melanotus* species, we saw no evidence of cross attraction of any other elaterid species to 13-14:OAc, nor did we see any evidence of attraction to any of the analogs or homologs that were tested. These data indicate that 13-14:OAc may be species-specific to *M. communis*, especially because a number of other *Melanotus* species were detected at our field sites using other monitoring methods. In fact, it was surprising that no other species were attracted to any of these compounds or blends given that many closely related elaterids have the same or similar pheromone components (Tóth 2013; Tolasch et al. 2013; König et al. 2015, 2016; Serrano et al. 2018). In sum, these results suggest that the pheromone components or pheromone blends of *Melanotus* and other click beetle species may be both quite diverse, but also narrowly tuned, with each species using either relatively unique compounds, or very specific ratios of compounds that may be shared by more than one species.

Our study is the first identification of a sex pheromone for a North American *Melanotus* species. Of the two other species in the genus for which pheromones have been fully reported, both from Japan, both produce esters of 12-carbon saturated and unsaturated alcohols. Thus, dodecyl acetate (12:OAc) was identified as the single sex pheromone component of *M. okinawensis* (Tamaki et al. 1986; Iwanaga and Kawamura 2000) , whereas a blend of (*E*)-9,11-dodecadienyl butanoate and (*E*)-9,11-dodecadienyl

hexanoate were identified as sex pheromone components of *M. sakishimensis* (Tamaki et al. 1990; Iwanaga and Kawamura 2000). (*E*)-9,11-Dodecadienyl butanoate and (*E*)-9,11-dodecadienyl hexanoate have also been identified in extracts of *M. tamsuyensis*, although bioassay data have not been reported (Yen and Chen 1998). Published observations in Europe suggest that *M. punctolineatus* (Pelerin) may use a tetradecenyl butyrate as a pheromone, whereas *M. rufipes* (Herbst) may use a tetradecadienyl butyrate (Tolasch et al. 2007). Pheromone glands of the west-Eurasian *M. fusciceps* and the Holarctic *M. castanipes* contained mixtures of >22 compounds, which included esters of 12-carbon and 14-carbon saturated and unsaturated alcohols, although the exact structures were not identified, nor were any bioassay data reported (Yatsynin et al. 1996). Thus, the compounds that we identified from *M. communis* in the present study are clearly similar to the pheromones or pheromone candidates found in other *Melanotus* species from Japan and Europe, especially the 14-carbon compounds of the west-Eurasian beetles. Further studies of the sex pheromones of additional *Melanotus* species should provide a more complete picture of the chemical diversity of the sex pheromones within this taxonomically large genus.

Insect sex pheromones have been exploited for pest management for more than 40 years, including the pheromones of click beetles. In Europe, pheromone-based methods have been particularly valuable for monitoring pest species (Kudryavtsev et al. 1993; Blackshaw and Vernon 2006; Vernon and Tóth 2007), as well as endangered non-pest species (Svensson et al. 2012). In addition, pheromone-based methods have been used to

monitor several invasive Eurasian click beetle species, i.e., *Agriotes* spp., that have become established in the U.S. and Canada (Vernon et al. 2001).

Sex pheromones have also been exploited in management of pest species, including *Melanotus okinawensis,* a major pest of sugarcane in Japan. Arakaki et al. (2008a) demonstrated that control of an isolated population of *M. okinawensis* was possible by mass trapping, using a high density of pheromone baited traps (10.6 traps/ha). The results of the 6-year study showed that the total number of hand-collected beetles and beetles captured in pheromone-baited traps in 2005 decreased by >85% and >70%, respectively, in comparison to numbers reported in 2000. A second study was conducted with another isolated population of *M. okinawensis* to determine if mating disruption could be used for control (Arakaki et al. 2008c). After the first year of this study, the numbers of beetles captured in pheromone traps decreased by ~96% in fields treated with pheromone. In addition, mean total trap catches decreased by 74% during the following 6 years in the study and significantly fewer females were mated in the treated area when compared to an untreated control area.

Click beetle pheromones also have the potentienal to be used in attract-and-kill strategies using entomopathogens (Kabaluk et al. 2015). Given that the pheromone of *M. communis* consists of a single component that can be readily synthesized, development of its pheromone for monitoring and control purposes may be both possible and economically feasible.

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Figures and Tables

Year tested	Compound(s) and ratio
2016, 2017	14:OAc
2016, 2017	13-14:OAc
2016, 2017	1:1 blend of 14:OAc to 13-14:OAc
2016, 2017	4:1 blend of 13-14:OAc to 14:OAc*
2016	1:1 blend of 12:OAc to 11-12:OAc
2017	1:1:1:1 blend of 14:OAc to 13-14:OAc to *E*11-14:OAc to *E*11,13-14:OAc
2016, 2017	2:1 blend of *E*11-14:OH to *E*11-14:OAc†

Table 4.1 Compounds and blends used for field bioassays of potential pheromone components of *Melanotus communis* in 2016 and 2017. *natural ratio found in SPME extracts from crushed abdomens of female *M. communis* beetles.†TABM lure.

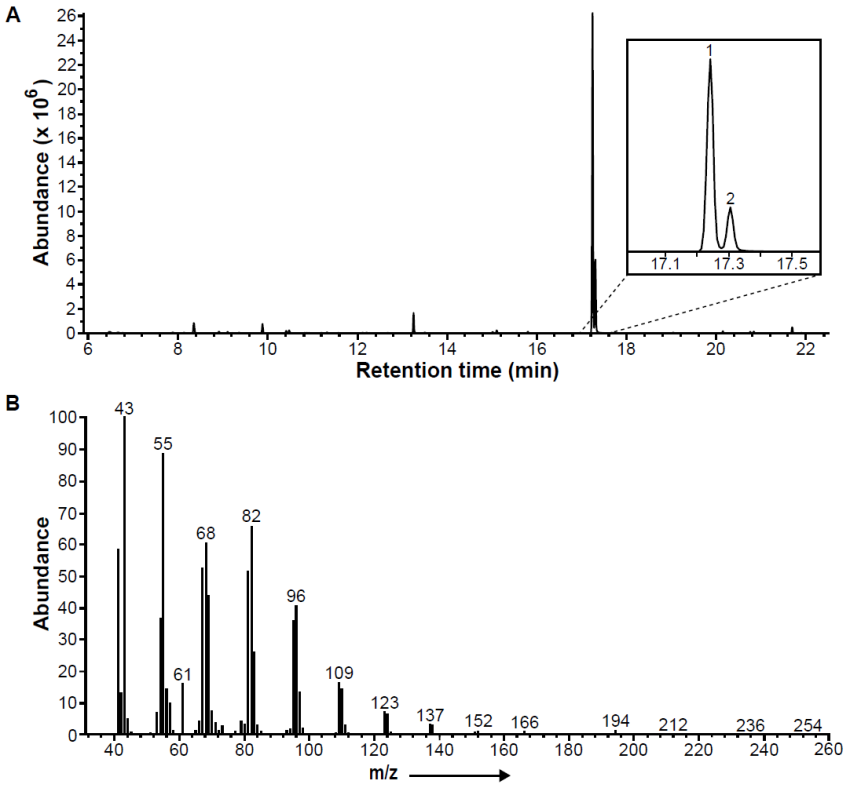

Figure 4.1 (A) Representative total ion chromatogram of an SPME extract of volatiles from the crushed abdomen of an adult female *Melanotus communis*, highlighting the pheromone candidates 13-tetradecen-1-yl acetate (peak 1) and tetradecyl acetate (peak 2); **(B)** EI mass spectrum (70 eV) of the female-produced 13-tetradecen-1-yl acetate

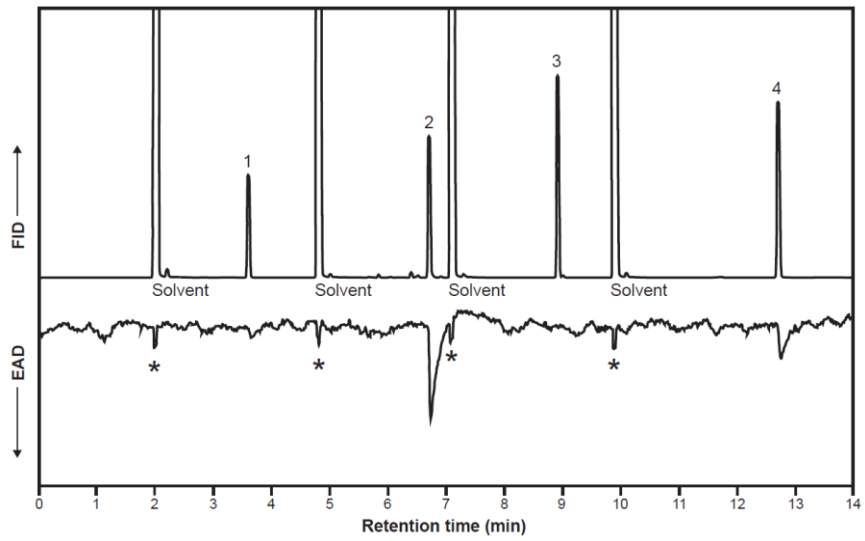

Figure 4.2 Representative GC-EAD analysis of alternating injections of 14:OAc (peak 1), 13-14:OAc (peak 2), *E*11-14:OAc (peak 3), and *E*11,13-14:OAc (peak 4), stimulating an antenna from a male *M. communis* beetle. Top trace: GC chromatogram; bottom, inverted trace: antennal responses. Large peaks in the GC trace are from the solvent used to make sequential injections, and the asterisks indicate the small responses elicited from the antenna by the solvent

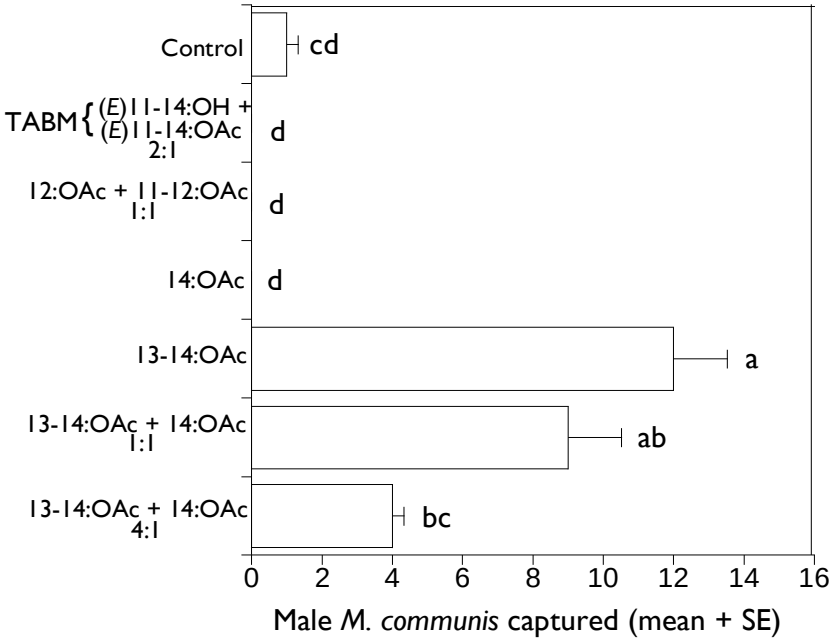

Figure 4.3 Mean total capture (+SE) per trap of male *Melanotus communis* beetles in traps baited with 12-carbon and 14-carbon acetates in 2016 (4 August – 13 September). Means with the same letter are not significantly different ($P > 0.05$).

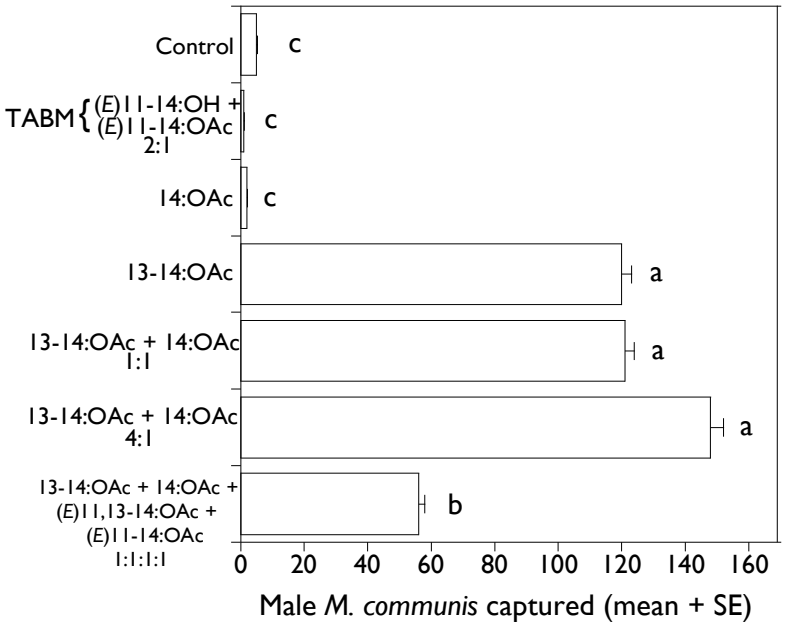

Figure 4.4 Mean total capture (+SE) per trap of male *Melanotus communis* beetles in traps baited with 14-carbon acetates in 2017. Means with the same letter are not significantly different ($P > 0.05$)

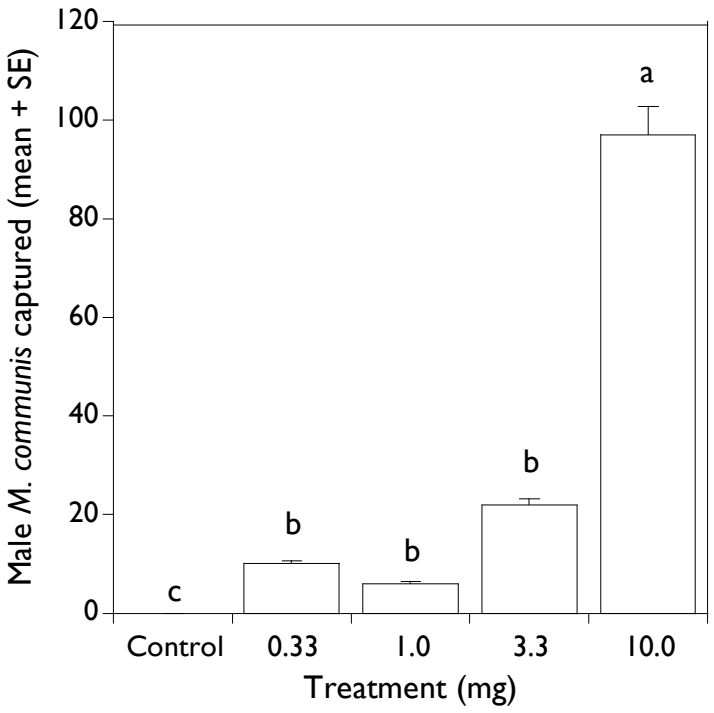

Figure 4.5 Mean total capture (+SE) per trap of male *Melanotus communis* beetles in traps baited with 0-10 mg doses over a semi-log range in 2018. Means with the same letter are not significantly different ($P > 0.05$)

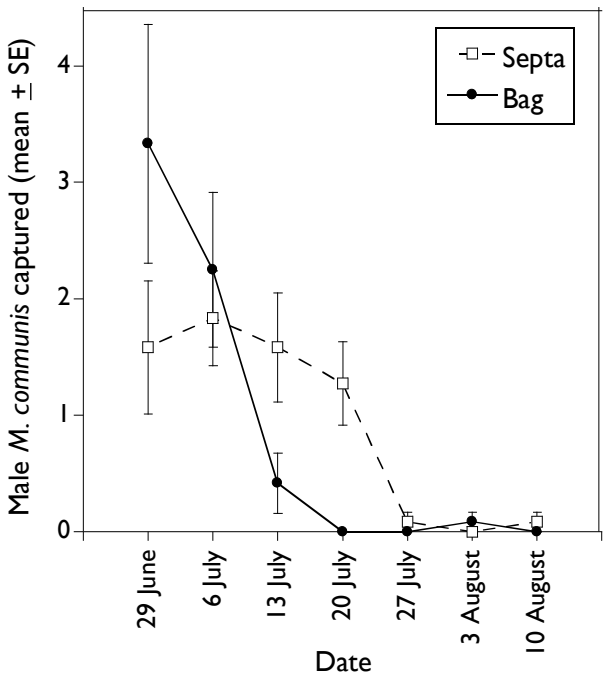

Figure 4.6 Weekly capture (mean ± SE) per trap of male *Melanotus communis* beetles in traps baited with 10 mg 13-14:OAc released from rubber septa or polyethylene bags.

Chapter 5: Sex Pheromone of *Idolus californicus* (Schaeffer) (Coleoptera: Elateridae)

Abstract

To date, most of all known click beetle (Coleoptera: Elateridae) pheromones have been identified from species that belong to the subfamily Elaterinae, specifically in the tribes Agriotini and Pomachiliini. While screening blends of some known elaterine sex pheromones in southern California, we discovered that *Idolus californicus* (Schaeffer) males were attracted to a blend that contained seven neryl esters. This suggested that one or more of the neryl esters might be the pheromone for this species. Electrophysiological assays with the blend of neryl esters determined that the antennae of males responded most strongly to neryl butyrate and neryl hexanoate. Further field bioassays with both neryl esters determined that males were specifically attracted by neryl hexanoate but not neryl butyrate. Subsequent analyses of extracts of volatiles from female beetles showed that the females actually produced neryl hexanoate and neryl octanoate. In field bioassays with the pheromone candidates, lures that contained neryl hexanoate alone or in blends with neryl octanoate attracted male beetles. Neryl octanoate was not attractive as a single compound and it neither synergized nor antagonized attraction to neryl hexanoate, even though the antennae of males responded to neryl octanoate in electrophysiological assays. Males of a *Dalopius* species also were captured in traps that baited with neryl octanoate, and electrophysiological assays showed that the antennae of *Dalopius* males responded to neryl octanoate butnot neryl hexanoate. This study is the first to demonstrate conservation of pheromone structures between European and North American elaterids.

Introduction

Many insects use pheromones as a means to communicate with each other, and these pheromones can be exploited for many purposes, including monitoring rare and endangered species (Larsson 2016), and for detection and management of pests. Pheromones in some insect taxa are highly conserved, with related species within a genus, tribe, or even subfamily tending to use different ratios and subsets of a core group of chemical components in their pheromone blends (Symonds and Elgar 2008; Löfstedt et al. 2016). Thus, field testing known pheromone components can provide strong leads as to what the pheromones of related species are likely to be. With such leads, i.e., knowing what types of compounds to be looking for, identification of the pheromones of these new species can be expedited. This strategy has been used successfully to identify pheromones or likely pheromones for several hundred species of longhorned beetles (Coleoptera: Cerambycidae) (reviewed in Millar and Hanks 2017; Silva et al. 2017).

Until 2018, no pheromones had been correctly identified for any of the ~1,000 North American click beetle species (Serrano et al. 2018). In addition, all known elaterid sex pheromones were from species in the subfamily Elaterinae, with the majority belonging to species in the genus *Agriotes* Eschscholtz (tribe Agriotini) (Tóth 2013). The first reported pheromones from *Agriotes* species, geranyl butyrate, geranyl isovalerate, (*E*,*E*)-farnesyl acetate, and neryl isovalerate, were identified from extracts of pheromone glands (Yatsynin et al. 1980) dissected from abdomens of female beetles (Ivastschenko and Adamenko 1980; Merivee and Erm 1993).

Since then, several more geranyl, farnesyl, and neryl esters have been identified as pheromones or likely pheromones for many *Agriotes* species across Europe (reviewed in Tóth 2013; Tóth et al. 2015). Additionally, these terpenoid esters have been identified as pheromones from species in the genera *Idolus* Desbrochers des Loges (Tolasch et al. 2013; König et al. 2015) and *Betarmon* Kiesenwetter (König et al. 2016), which belong to the tribe Pomachiliini, closely related to the Elaterini.

Although the existing data for elaterid pheromones is from less than 50 species, patterns of pheromone diversity within the family are beginning to emerge. For example, in the subfamily Cardiophorinae, two species in the genus *Cardiophorus* Eschscholtz were shown to use the same female-produced sex pheromone, methyl (3*R*,6*E*)-2,3-dihydrofarnesoate (Serrano et al. 2018). Furthermore, because of the conservation of pheromone structures within closely related species, elaterid pheromones have been used as taxonomic characters in a few instances (Tolasch 2008; Vuts et al. 2012; König et al. 2015). Therefore, we hypothesized that the known pheromones of European click beetle species might be conserved within closely related taxa, including North American congeners. To test this hypothesis, we decided to field screen many of the known and conserved elaterid pheromones reported in Tóth (2013) with the hope that this provide leads to possible pheromones for any species that were attracted to these compounds. There is also some precedent for this strategy, which had been used to help identifiy pheromones for European elaterids such as *Agriotes sordidus* Illiger (Tóth et al. 2002; Tóth et al. 2015) and *Agriotes proximus* Schwarz (Tóth et al. 2008; Vuts et al. 2012).

In 2015, we conducted field trials with some known elaterid pheromones, including blends of geranyl and neryl esters, in the San Bernardino National Forest in southern California. During this preliminary screening, we observed that a significant number of click beetles were specifically attracted to the blend of neryl esters. These beetles were all identified as *Idolus californicus* (Schaeffer), and all were males (Fig. 5.1). Female beetles from related European species in the tribes Agriotini and Pomachiliini are known to produce blends of two of the terpenoid esters in the neryl ester blend that we tested (reviewed in Tóth 2013; Tolasch et al. 2013; König et al. 2015, 2016). Therefore, we hypothesized that one or more of the neryl esters in the test blend were responsible for the attraction of *I. californicus* males.

Thus, our goal was to follow up on these field screening results by identifying the actual female-produced sex pheromone component(s) of *I. californicus*. The specific objectives of this study were: (1) to determine which of the seven neryl esters were necessary for attraction; (2) to analyze female-produced volatiles to determine if they contained one or more neryl esters and/or other compounds; (3) to field test the female-produced pheromone candidates; and (4) to field test different ratios of neryl hexanoate and neryl octanoate, the two compounds identified in extracts from female *I. californicus*.

Materials and Methods

Insects Adult *Idolus californicus* beetles were collected at four sites in the San Bernardino National Forest in San Bernardino Co., CA, USA. Two of the sites were near Jenks Lake as described in Serrano et al. (2018), and the other two sites were near

Crestline, CA as described in Serrano et al. (2019). Adult female *I. californicus* were collected June 20–26 2018 as random captures in flight intercept traps. Beetles were sexed by gently squeezing the abdomens of the beetles to extrude the genitalia. Mating status, age, and other life history factors of the beetles were unknown. Voucher specimens of *Idolus californicus* have been deposited in the Entomology Research Museum at UC Riverside.

Collection and Analyses of Extracts Volatiles were collected from individual crushed abdomens (n=2) of freeze-killed *I. californicus* females using solid phase microextraction (SPME) methods as reported in Serrano et al. (2018). SPME extracts were analyzed with an HP 6890 gas chromatograph (Agilent, Santa Clara, CA, USA) fitted with a DB-17 column (30 m × 0.25 mm ID × 0.25 μm film; J&W Scientific, Folsom, CA, USA), coupled to an HP 5973 mass selective detector. The polydimethylsiloxane SPME fibers were desorbed in the injector port (250 °C) in splitless mode for 30 s prior to starting the run. The temperature program used was 100 °C/1 min, then increased at 10 °C/min to 280 °C. Compounds were tentatively identified by mass spectral interpretation and matches with database spectra (NIST 14, Agilent), and then confirmed by matching retention times and mass spectra with those of authentic standards.

Gas Chromatography-Electroantennogram Detection (GC-EAD) Analyses were conducted as described in Serrano et al. (2018) with a Hewlett-Packard 5890 GC (Agilent) fitted with either a DB-17 column (30 m × 0.25 mm ID × 0.25 μm film; J&W

Scientific) or a DB-5 column (30 m × 0.25 mm ID × 0.25 μm film; J&W Scientific). The temperature program used for analyses was either 50 °C/1 min, then 10 °C/min to 280 °C or 100 °C/1 min, then 10 °C/min to 280 °C, with an injector temperature of 250 °C. SPME analyses were conducted as described above, whereas analyses of standards were carried out by injecting 1 μl aliquots of hexane solutions in split mode.

Chemicals Blends of neryl and geranyl esters were prepared for field screening. Thus, a blend of 2 mmol each of acetyl, butyryl, isobutyyrl, isovaleryl, hexanoyl, octanoyl, and decanoyl chlorides was added dropwise to an ice-bath cooled solution of nerol (1.23 g, 8 mmol), pyridine (0.8 ml, 10 mmol), and ~50 mg dimethylaminopyridine catalyst in 50 ml of dry ether. The resulting mixture was warmed to room temperature and stirred 3 h. Glycerin (2 ml) was then added, and the mixture was stirred for an additional 1 h. 50 ml of saturated aqueous $NaHCO_3$ was then added, the mixture was stirred 10 min, then the layers were separated. The ether layer was washed successively with saturated aqueous $NaHCO_3$, twice with 1M HCl, once with water, and once with brine, then dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by vacuum flash chromatography on silica gel, eluting with 5% EtOAc in hexane. The purified ester blend was used directly for field experiments. The reaction was repeated, substituting geraniol for nerol, to generate the analogous geranyl ester blend.

Neryl butyrate was synthesized as follows. Butyryl chloride (4.14 ml, 40 mmol) was added dropwise to an ice-bath cooled solution of nerol (5.39 g, 35 mmol), pyridine (3.7 ml, 47 mmol) and dimethylaminopyridine catalyst (100 mg) in 100 ml methylene

chloride. After the addition was complete, the mixture was removed from the ice bath and stirred 3 h at room temp. Residual butyryl chloride was then destroyed by addition of methanol and stirring for an additional 3 h. The resulting mixture was then extracted sequentially with water, 1M HCl, saturated $NaHCO_3$, and brine, then concentrated to an odorous oil. The product was purified by Kugelrohr distillation (oven temp ~108 ºC, 0.7 mm Hg), yielding neryl butyrate (6.48 g, 83 %), which was >95% pure by GC. ^{1}H NMR, ^{13}C NMR, and mass spectral data agreed with those previously reported in Tolasch et al. (2010). Neryl hexanoate and neryl octanoate were prepared using the same esterification methods as described above, substituting hexanoyl or octanoyl chloride respectively in place of butyryl chloride. Neryl hexanoate was used without further purification and was >95% pure by GC, and neryl octanoate was purified by Kugelrohr distillation (oven temp ~116 ºC, 0.3 mm Hg). The purified ester was >98% pure by GC. ^{1}H NMR, ^{13}C NMR, and mass spectral data for neryl hexanoate and neryl octanoate agreed with those previously reported in Tolasch et al. (2013).

Field Screening of Geranyl and Neryl Esters The first field bioassays testing known elaterid pheromones were carried out in June 2015 at the two Jenks Lake sites (described above). Compounds included mixtures of geranyl esters (geranyl acetate, geranyl isobutyrate, geranyl butyrate, geranyl isovalerate, geranyl hexanoate, geranyl octanoate, and geranyl decanoate) and neryl esters (neryl acetate, neryl isobutyrate, neryl butyrate, neryl isovalerate, neryl hexanoate, neryl octanoate, and neryl decanoate). Dispensers were prepared as described in Tolasch et al. (2007) from 0.2 ml PCR tubes (Molecular

BioProducts Inc., #3412) that had been pierced with a size 3 insect pin at the front side 2 mm below the lid. Dispensers were loaded with 0.25 ml of a 10 mg/ml solution (in hexane) of the compounds. Control lures contained hexane only. One set of treatments was tested at each of the two locations near Jenks Lake site (N=2). Traps were checked twice weekly, with lures replaced weekly. Black cross-vane panel traps (Alpha Scents, Portland, OR, USA) coated with Fluon and hung on 2 m tall PVC pipe stands were used at all sites. The inner walls of the collecting bucket of each trap were coated with Fluon to retain trapped beetles, which were live trapped so that they could be brought back to the laboratory for sex determination, and subsequent use in coupled gas chromatography-electroantennogram detection (GC-EAD) analyses. After obtaining results from GC-EAD analyses, neryl hexanoate was then tested in the field as a single compound.

Field Bioassays Testing Neryl Butyrate and Neryl Hexanoate In 2016, an experiment was carried out to follow up on the 2015 results by testing the responses of *I. californicus* males to different blends of neryl butyrate and neryl hexanoate, the two compounds in the neryl ester blend which had elicited strong antennal responses in GC- EAD trials. The experiment ran from 6 – 27 June 2016, at the two Jenks Lake locations. Modified intercept traps described in Serrano et al. (2018) were used, and traps were baited with lures prepared as described above. Lures were baited with a 1:1 (10 mg each) and a 4:1 (10 mg and 2.5 mg, respectively) ratio of neryl hexanoate to neryl butyrate. The 4:1 ratio was chosen based on the average intensity of male antennal responses to each compound in GC-EAD analyses. One set of treatments was tested at each of the two locations

described above, with treatments initially assigned randomly to traps. Traps were placed 10-15 m apart and were checked twice weekly, resulting in 8 temporal replicates (see Statistical Analyses below). Lures were replaced weekly at which time the trap order was rotated one position.

Another experiment was set during 12 May – 26 June 2017 to test neryl hexanoate and neryl butyrate as individual compounds and in different blends. Lures consisted of 5 × 7.5 cm (~0.05mm thickness) low-density polyethylene zipper seal bags (Fisher Scientific, #01–816-1A), filled with 1 ml solutions of test compounds in isopropanol. For this experiment, the treatments were: 1) neryl butyrate (10 mg/ml); 2) neryl hexanoate (10 mg/ml); 3) 4:1 ratio of neryl hexanoate to neryl butyrate (10 and 2.5 mg/ml, respectively); 4) 10:1 ratio of neryl hexanoate to neryl butyrate (10 and 1 mg/ml, respectively); and 5) isopropanol control. Traps, trap location, and trap and lure maintenance were as described above in the field bioassays conducted in 2016, resulting in 20 temporal replicates.

Field Bioassays Testing Pheromone Candidates In 2018, field experiments were run to test the pheromone candidates neryl hexanoate and neryl octanoate, which had been found in SPME extracts from crushed abdomens of *Idolus californicus* females. The experiments were run at the Jenks Lake sites from 8 May to 15 June, and the treatments consisted of: 1) neryl hexanoate (10 mg/ml); 2) neryl octanoate (10 mg/ml); 3) 100:28.5 ratio of neryl hexanoate to neryl octanoate (10 and 2.85 mg/ml, respectively); and 4) isopropanol control. The 100:28.5 ratio represented the natural ratio of the two neryl

esters found in SPME extracts of the crushed abdomens from *I. californicus* females. Traps, lures, trap locations, and maintenance of traps and lures were as described above in field bioassays conducted in 2017, resulting in 22 temporal replicates.

In a final field trial for *I. californicus*, we tested different blends of neryl octanoate in semi-log steps (0.1, 0.33, 1, 3.33, and 10 mg) in combination with a fixed dose of neryl hexanoate (10 mg), all in 1 ml isopropanol. Controls consisted of isopropanol alone. This experiment was conducted 14 May to 14 June 2018 at the two Crestline sites (i.e. one replicate of treatments per site). Traps, lures, and maintenance were as described above, resulting in 10 temporal replicates.

Statistical Analyses Replicates for each field bioassay were based on both spatial and temporal replicates, with temporal replicates being the number of times the traps were checked. Statistical analyses were conducted with R version 3.4.2, used with RStudio version 1.1.383 (R-Development-Core-Team 2017; RStudioTeam 2016). Differences among treatment means were first assessed by the Kruskal-Wallis rank sum test, followed by Dunn's multiple comparison test using the FSA package (Dunn 1964; Ogle et al. 2019).

Results

Identification of Neryl Hexanoate as a Sex Attractant The first indication that one or more neryl esters might be sex pheromone components of *I. californicus* was obtained during field screening of the blend of neryl esters in 2015 at the two Jenks Lake sites.

Traps baited with the neryl ester mix attracted large numbers of male *I. californicus*. In GC-EAD analyses with the same neryl ester blend, neryl butyrate and neryl hexanoate both elicited responses from antennae of males (Fig. 5.2), with the responses to neryl hexanoate being the strongest and most consistent. Neryl hexanoate was then field tested as a single component. At the first trap check, two traps contained a total of 43 beetles. However, due to a forest fire near the field site, traps were inaccessible for 2 wk. No additional beetles were caught after access to the field site was restored, either due to being wiped out by the fire, or due to the seasonal activity period ending.

In follow-up field trials in 2016 at the same two sites, traps baited with the 4:1 ratio of neryl hexanoate to neryl butyrate caught significantly more beetles than controls ($N = 8$; Kruskal-Wallis $\chi^2_{(2)} = 14.89$, $P < 0.001$; Dunn's test $P < 0.001$) (Fig. 5.3), whereas the 1:1 blend was not significantly different from either the 4:1 blend or the controls (Fig. 5.3; Dunn's test $P > 0.05$). The total numbers of *I. californicus* males captured for all treatments were: 56 in the 4:1 blend of neryl hexanoate to neryl butyrate, 15 in the 1:1 blend of neryl hexanoate to neryl butyrate, and 0 in the controls. In GC-EAD analyses with standards of neryl butyrate and neryl hexanoate, an antenna from a male *I. californicus* responded more strongly to neryl hexanoate than neryl butyrate (Fig. 5.4).

In 2017, traps baited with neryl hexanoate captured significantly more male *I. californicus* beetles than the traps baited with neryl butyrate alone and the control (Fig. 5.5; $N = 22$; Kruskal-Wallis $\chi^2_{(4)} = 67.95$, $P < 0.001$; Dunn's test $P < 0.001$). There was no significant difference in the mean number of *I. californicus* beetles captured between

the three neryl hexanoate treatments (Dunn's test $P > 0.05$). The total trap catches for all treatments were: 1 in neryl butyrate, 1215 in neryl hexanoate, 1144 in the 4:1 blend of neryl hexanoate to neryl butyrate, 1421 in the 10:1 blend of neryl hexanoate to neryl butyrate, and 0 in the controls.

Identification of the Sex Pheromone In 2018, two females of *I. californicus* were collected from the Crestline field sites. GC-MS analyses of the SPME-collected headspace volatiles from crushed female abdomens revealed two major peaks in the total ion chromatograms (Fig. 5.6a). The two compounds were tentatively identified from matches of their mass spectra with those of neryl hexanoate and neryl octanoate. The identifications were confirmed by matching their retention times and mass spectra with those of authentic standards. Additionally, in GC-EAD analyses testing neryl hexanoate and neryl octanoate standards, both compounds elicited responses from antennae of males (Fig. 5.6b), with the responses to neryl hexanoate being the strongest and most consistent.

Field Bioassays with Pheromone Candidates Traps baited with neryl hexanoate and the 100:28.5 blend of neryl hexanoate to neryl octanoate captured significantly more *I. californicus* males (911 and 1560 total, respectively) than traps that were baited with neryl octanoate or the controls (9 and 0 total, respectively) ($N = 20$; Fig. 5.7; Kruskal-Wallis, $\chi^2_{(3)} = 63.85$, $P < 0.001$; Dunn's test, $P < 0.001$). There was no significant difference in the mean number of *I. californicus* male beetles captured between the two treatments that contained neryl hexanoate (Fig. 5.7; Dunn's test $P > 0.05$). Traps baited

with neryl octanoate and the 100:28.5 blend of neryl hexanoate to neryl octanoate also captured males of a *Dalopius* species. For this species, there were significant differences in the mean numbers of males captured between the three treatments and the control (Fig. 5.8a; Kruskal-Wallis, $\chi^2_{(3)} = 53.53$, $P < 0.001$). There was no significant difference between traps baited with neryl octanoate or the blend of neryl octanoate and neryl hexanoate (Dunn's test, $P > 0.05$). GC-EAD analyses showed that the antennae from *Dalopius* males responded to neryl octanoate but not neryl hexanoate (Fig. 5.8b).

Field bioassays testing the response of *I. californicus* males to semi-log blends of neryl octanoate with neryl hexanoate showed that there were no significant differences between the number of beetles captured in all pheromone-baited treatments ($N = 10$; Fig. 5.9; Kruskal-Wallis, $\chi^2_{(5)} = 19.884$, $P < 0.01$; Dunn's test $P > 0.05$).

Discussion

Males of the click beetle species *Idolus californicus* had been caught in preliminary field bioassays testing combinations of known elaterid pheromones which included seven neryl esters. Of the seven esters in the blend, GC-EAD analyses showed that the antennae of males reliably responded to only two of the components, neryl butyrate and neryl hexanoate. Follow-up field bioassays conducted in 2016 and 2017 were intended to determine if *I. californicus* males were attracted to neryl butyrate and neryl hexanoate as single components, or mixed in different ratios. The results from those bioassays demonstrated that neryl hexanoate as a single component appeared to be both necessary and sufficient for attraction. Neryl butyrate had no apparent effect on attraction when

blended with neryl hexanoate, and it was not attractive as a single component, even though it elicited antennal responses from antennae of males in GC-EAD analyses. These data provided support for neryl hexanoate being the major and possibly only pheromone component for this species.

When comparing the results from bioassays conducted in 2016 against those conducted in 2017-2018, there were considerable differences in the average and total numbers of male beetles captured in traps. This was most likely due to changing the release devices from small plastic centrifuge tubes with a pinhole in them to low density polyethylene sachets. Although we did not directly compare the release rates and attraction of each lure device in the field, the much thinner walled and higher surface area sachets almost certainly had higher release rates than the smaller and much thicker walled plastic tubes. In addition, the populations of *I. californicus* may have been affected by the large forest fire that had burned within 2 km of the field sites at Jenks Lake.

Analyses of volatiles from crushed female abdomens revealed that females do not produce neryl butyrate, but they do produce neryl hexanoate and neryl octanoate. These data supported our previous field bioassay and GC-EAD results which had suggested that neryl hexanoate was an important component of the female-produced sex pheromone of *I. californicus.* Although neryl octanoate elicited antennal responses from males and was present in volatiles collected from the crushed abdomens of females, in field bioassays, it was not attractive as a single component, nor did it appear to have any effect on attraction when presented in blends with neryl hexanoate.

As mentioned above, GC-EAD analyses of neryl butyrate, neryl hexanoate, and neryl octanoate showed that males can detect all three compounds but only neryl hexanoate was attractive in the field. The antennal responses to neryl butyrate and neryl octanoate may have been due to the structural similarities between the three neryl esters. Alternatively, neryl butyrate and neryl octanoate may play a role in maintaining reproductive isolation among closely related elaterine species that share one or more neryl esters as pheromone components, such as the *Dalopius* species that was attracted to traps baited with neryl octanoate. However, neryl octanoate can only be labeled as a male sex attractant for the *Dalopius* species because we were unable to collect females of this species, and therefore could not confirm that females actually produce neryl octanoate, and possibly additional compounds.

Both neryl hexanoate and neryl octanoate have previously been identified as pheromone components for an undescribed *Idolus* species from Europe (Tolasch et al. 2013). However, it is unclear if both neryl esters were required to attract males of this species, or if males were only attracted to one of the two components identified in extracts of the pheromone glands of females. In addition, neryl hexanoate, neryl octanoate, and neryl decanoate have been identified as pheromone components for *Betarmon bisbimaculatus* (Fabricus), and results from field bioassays demonstrated that all three esters were required for significant attraction of males, and that only neryl hexanoate was slightly attractive as a single component (König et al. 2016).

In summary, our results provide evidence that neryl hexanoate is the major and only female-produced sex pheromone component of *Idolus californicus*. This compound

had been identified a sex pheromone component for a few European click beetle species (Tolasch et al. 2013; König et al. 2016), and we have now confirmed that it is a pheromone component for a related North American species. It remains to be determined whether neryl octanoate is the pheromone for the *Dalopius* species, and if it will be another conserved pheromone shared by European and North American elaterids. To our knowledge, this is the first instance in which pheromones from European elaterids have been shown to be conserved in related species from other continents. The work presented here is also the fourth pheromone identification for North American click beetle species.

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Figures

Figure 5.1 Dorsal image of an *Idolus californicus* male.

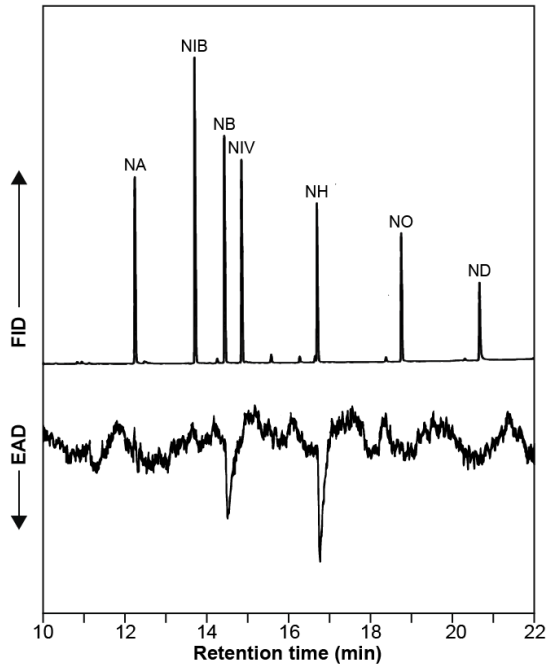

Figure 5.2 Representative GC-EAD analyses (on a DB-17 column) of neryl acetate (NA), neryl isobutyrate (NIB)**,** neryl butyrate (NB), neryl isovalerate (NIV), neryl hexanoate (NH)**,** neryl octanoate (NO), and neryl decanoate (ND) stimulating an antenna from a male *I. californicus*. Top trace shows the GC chromatogram and the bottom, inverted trace shows the responses from the antenna of a male.

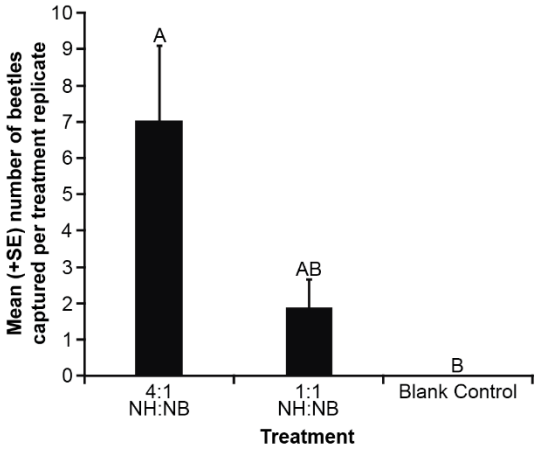

Figure 5.3 Mean (+1 SE) numbers of *I. californicus* males caught in traps baited with a 4:1 and 1:1 ratio of neryl hexanoate (NH) to neryl butyrate (NB) (9 – 27 June 2016 at the Jenks Lake sites). Means with the same letter are not significantly different (Dunn's test, $P > 0.05$).

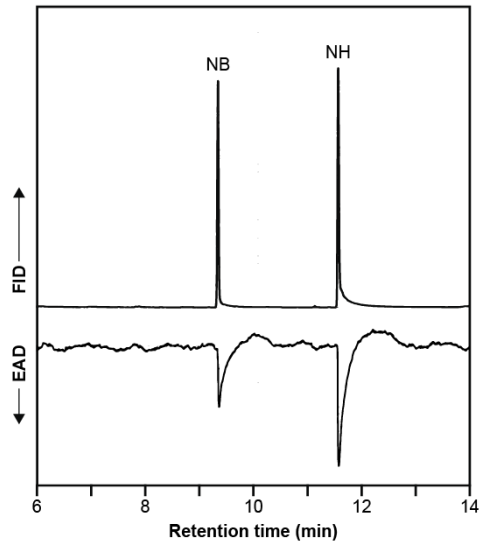

Figure 5.4 Representative GC-EAD analyses (on a DB-17 column) of neryl butyrate (NB) and neryl hexanoate (NH) stimulating an antenna from a male *I. californicus*. Top trace shows the GC chromatogram and the bottom, inverted trace shows the responses from the antenna of a male beetle.

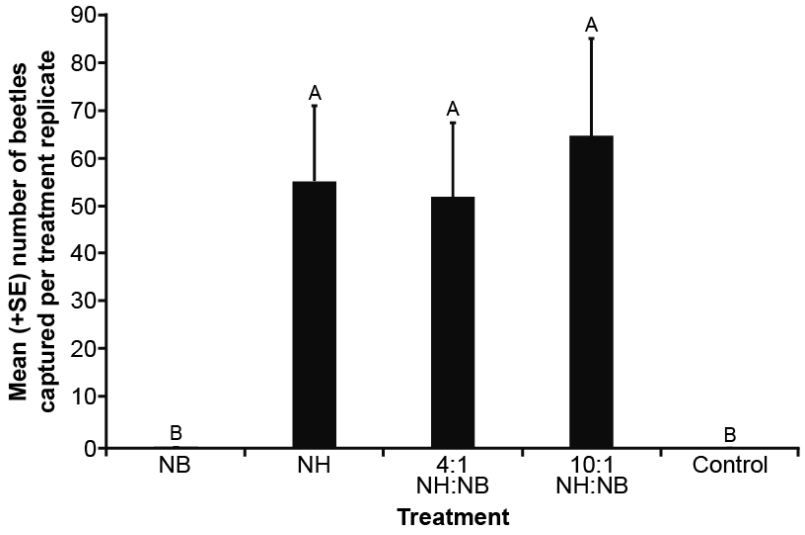

Figure 5.5 Mean (+1 SE) numbers of *I. californicus* males caught in traps baited with neryl butyrate (NB), neryl hexanoate (NH), a 4:1 ratio of NH:NB, and a 10:1 ratio of NH:NB in isopropanol (15 May – 26 June 2017 at the Jenks Lake sites). The solvent control was isopropanol. Means with the same letter are not significantly different (Dunn's test, $P > 0.05$).

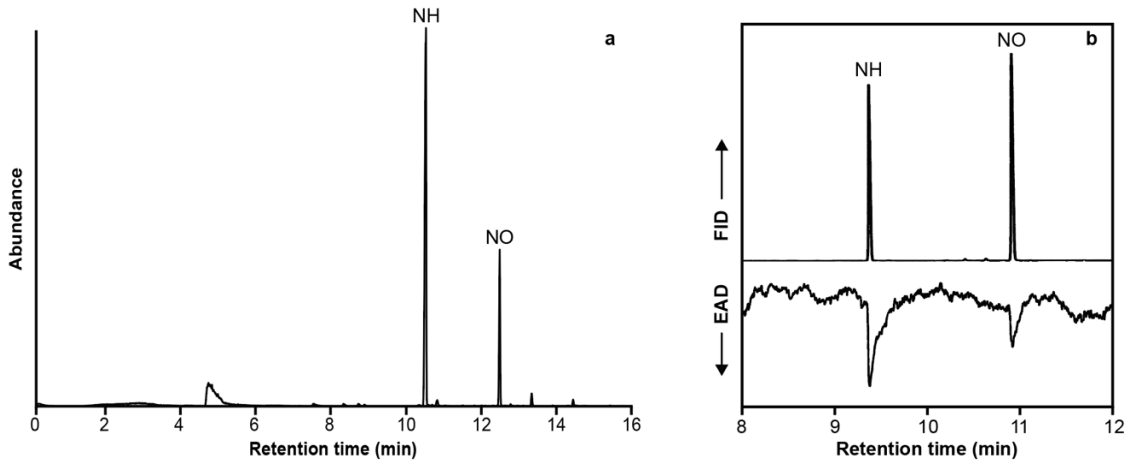

Figure 5.6 (a) Representative total ion chromatogram (on a DB-17 column) of an SPME extract of volatiles from the crushed abdomen of an adult female *I. californicus*. The pheromone candidates are highlighted as neryl hexanoate (NH) and neryl octanoate (NO). **(b)** Representative GC-EAD analyses (on a DB-5 column) of neryl hexanoate (NH) and neryl octanoate (NO) stimulating an antenna from a male *I. californicus.* Top trace shows the GC chromatogram and the bottom, inverted traces show the responses from the antenna of a male beetle.

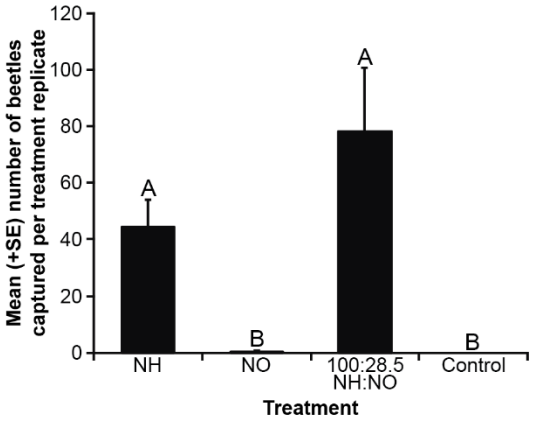

Figure 5.7 Mean (+1 SE) numbers of *I. californicus* males caught in traps baited with neryl hexanoate (NH), neryl octanoate (NO), and a 100:28.5 ratio of NH:NO (11 May – 15 June 2018 at the Jenks Lake sites). The solvent control was isopropanol. Means with the same letter are not significantly different (Dunn's test, $P > 0.05$).

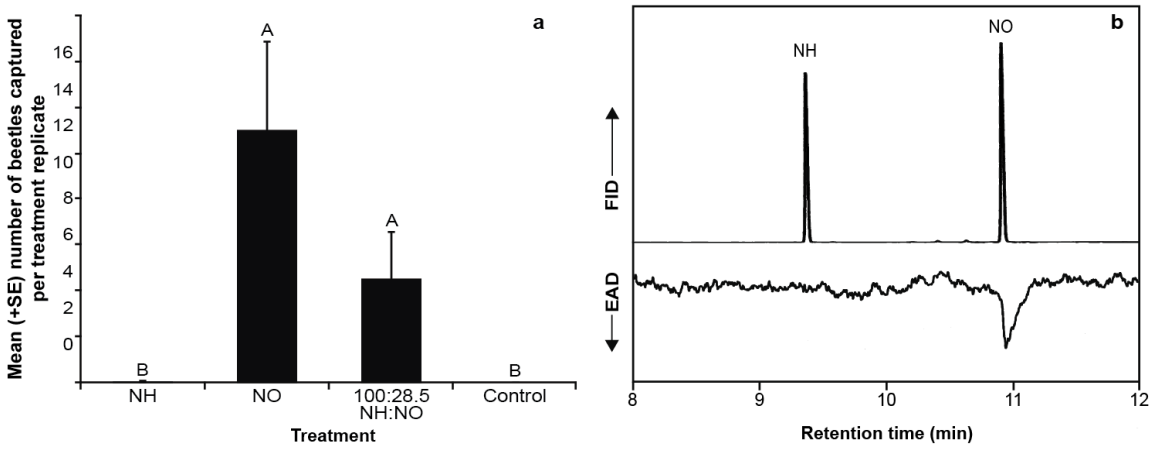

Figure 5.8 (a) Mean (+1 SE) numbers of *Dalopius* sp. males caught in traps baited with neryl hexanoate (NH), neryl octanoate (NO), and a 100:28.5 ratio of NH:NO (11 May – 15 June 2018 at the Jenks Lake sites). The solvent control was isopropanol. Means with the same letter are not significantly different (Dunn's test, $P > 0.05$). **(b)** Representative GC-EAD analyses (on a DB-5 column) of neryl hexanoate (NH) and neryl octanoate (NO) stimulating an antenna from a *Dalopius* sp. male. Top trace shows the GC chromatogram and the bottom, inverted trace shows the responses from the antenna of a male beetle.

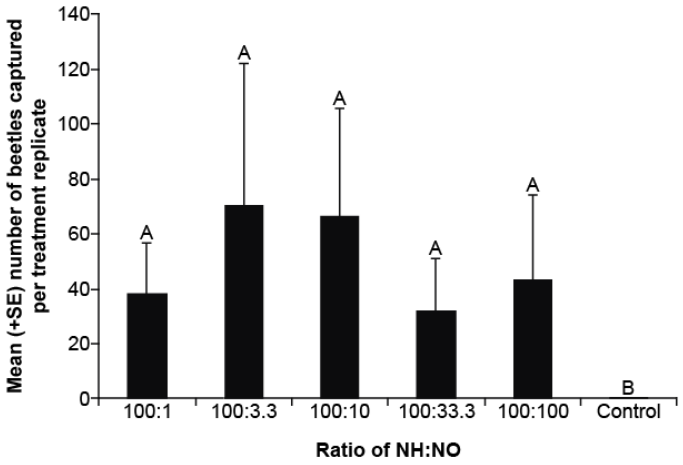

Figure 5.9 Mean (+1 SE) numbers of *I. californicus* males caught in traps baited with lures in which the dose of neryl hexanoate (NH) was held constant, and different amounts of neryl octanoate were added following a semi-log scale (17 May – 14 June 2018 at the Crestline sites). The solvent control was isopropanol. Means with the same letter are not significantly different (Dunn's test, $P > 0.05$).

Chapter 6: Conclusion

Click beetles (Coleoptera: Elateridae) are a diverse and cosmopolitan family of beetles that are well known for their clicking mechanism. There are approximately 1,000 described click beetle species in the United States (Bouchard et al. 2017), but not much is known about even the basic biology of a majority of these species. Some elaterids are serious pests in agricultural systems, whereas others natural landscapes where they are predaceous on other arthropods, or saprophagous, playing important roles in breakdown of woody debris and forest turnover.

Many insects use chemical signals to communicate with each other, and these compounds, known as pheromones, can be exploited for monitoring rare species and the detection and management of pests. Indeed, insect pheromones now are key elements in many modern integrated pest management (IPM) programs, being used as tools to detect, monitor, attract-and-kill, or disrupt the mating of pest insects. In Europe and Asia, effective methods of monitoring and controlling adult click beetles have been developed, based on traps baited with the attractant pheromones that the female beetles produce to attract males for mating (Svensson et al. 2012; Tóth 2013; Traugott et al. 2015). Before the start of my dissertation research, no pheromones had been identified for any of the ~1,000 North American click beetle species, and worldwide, pheromones had only been identified from species in the subfamily Elaterinae, despite the major potential for exploiting these naturally produced compounds in IPM and monitoring programs for elaterids in general.

The main goals of this dissertation were to identify the first pheromones for North American click beetles, and to establish protocols that would allow for efficient and successful pheromone identifications in the future. The work presented here has

expanded our knowledge about click beetle pheromone communication not only within North American, but also globally.

My first two identifications of sex pheromones and sex attractants were due to the serendipitous discovery that *Cardiophorus tenebrosus* and *C. edwardsi* males were attracted to the known longhorned beetle (Coleoptera: Cerambycidae) pheromone, fuscumol acetate [(*E*)-6,10-dimethylundeca-5,9-dien-2-yl acetate] (Hanks and Millar 2016). In the early stages of this work, it was unclear whether fuscumol acetate was indeed produced by the females of both *Cardiophorus* species, but before any females were obtained for analyses, we were able to confirm that males of both species were specifically attracted to the (*R*)-enantiomer of fuscumol acetate. In addition, the (*S*)-enantiomer of fuscumol acetate did not appear to be detected or to have an antagonistic effect when present in a racemic mixture with the (*R*)-enantiomer.

When females of both *Cardiophorus* species were collected, analyses showed that they produced (*R*)-methyl dihydrofarnesoate, which was confirmed as the sex pheromone in bioassays. This compound had not been reported previously from the family Elateridae, however it does share some structural similarities with some of the terpenoid esters which had been previously identified as elaterid pheromone components. Comparison of the structures of (*R*)-fuscumol acetate and (*R*)-methyl dihydrofarnesoate revealed that the two compounds shared substantial structural similarities which may explain the responses of males of both *Cardiophorus* species to (*R*)-fuscumol acetate. The findings presented in Chapter 2 also represent the first successful identifications of sex pheromones and sex attractants for any North American click beetle species, and for

any species in the subfamily Cardiophorinae. Future work on these two *Cardiophorus* species might investigate how these two species remain reproductively isolated; some possible explanations are discussed in Chapter 2.

The work described in Chapter 3 was meant to explore why both *C. tenebrosus* and *C. edwardsi* responded so well to (*R*)-fuscumol acetate, by testing the responses of males of both species to three terpenoid esters that were structurally similar to (*R*)-methyl dihydrofarnesoate and (*R*)-fuscumol acetate. The results from this study confirmed that chirality and the three-dimensional spatial relationships within the molecules were all important factors for recognition of the pheromones or analogs by receptors of these species. Originally, we had postulated that the terpenoid region of the esters was not important for pheromone recognition, due to the lack of responses to the (*S*)-enantiomers of both fuscumol acetate and methyl dihydrofarnesoate, which have exactly the same terpenoid chain as the natural pheromone, but differ in the chirality of the ester function. Contrary to our expectations, we found that the terpenoid chain structure, and in particularl, the number of isoprenoid units, was also a crucial factor for bioactivity. Specifically, the shorter terpenoid analogs elicited neither electroantennogram nor behavioral responses from *Cardiophorus* males, despite having the same or similar polar head groups to (*R*)-methyl dihydrofarnesoate or (*R*)-fuscumol acetate.

However, one of the analogs, fuscumol propionate, was found to be a “super stimulus” for *C. edwardsi* when compared to the actual pheromone, attracting ~26 times as many males as the actual pheromone in field trials. In these trails, the release rates of fuscumol propionate and methyl dihydrofarnesoate were similar, so differences in release

rates could not explain the hyperactivity of fuscumol propionate. This level of hyperactivity stimulated by a pheromone mimic is unprecedented within the insect pheromone literature. Even more inexplicable was the fact that in electroantennogram assays, antennae from *C. edwardsi* males responded significantly less to fuscumol propionate than to the pheromone. Thus, it remains unclear which step or steps in the sequence from pheromone capture by the antenna through to behavioral output is responsible for this hyperstimulatory effect.

The next successful pheromone identification was for a pest species from the subfamily Elaterinae, the corn wireworm, *Melanotus communis*. This species is a major pest of multiple crops in the eastern United States and is of major concern to corn, potato, and sugarcane growers due to the lack of effective management tools for this pest. Tetradecyl acetate and 13-tetradecenyl acetate were identified as pheromone candidates from extracts of female beetles. These compounds are related to but not identical to pheromone components that had been identified from Japanese *Melanotus* species (reviewed in Tóth 2013). Field bioassays demonstrated that only the major component, 13-tetradecenyl acetate, was bioactive, which suggests that tetradecyl acetate may play a role in maintaining reproductive isolation from other sympatric *Melanotus* species.

Compounds that were structurally related to 13-tetradecenyl acetate also were tested in bioassays, including the pheromone blend of the tufted apple budmoth, (*E*)-11-tetradecenyl acetate and (*E*)-11-tetradecenyl, due to older reports of these compounds being attractive to *M. depressus* (Weires 1976; Brown and Keaster 1983). The tufted apple budmoth lure was not attractive to *M. communis* males, and (*E*)-11-tetradecenyl

acetate did not elicit any responses from antennae of males. Other analogs that were tested in field bioassays included compounds that closely resembled the pheromones of both *M. communis* and their Japanese congeners. One of the analogs, (*E*)-11,13-tetradecadienyl acetate, elicited antennal responses from *M. communis* males, but the responses may have been a result of the structural similarity between the analog and 13-tetradecenyl acetate.

Another interesting result from these field bioassays was that males from seven sympatric *Melanotus* species were not attracted to any of the compounds that were tested. This was surprising because the analogs were structurally similar to the pheromone components of *M. communis* and the three *Melanotus* species from Japan. This finding suggests that pheromones may not be well conserved within this large genus, and that other species may utilize pheromones with entirely novel structures. Alternatively, pheromones of these species may consist of blends of compounds, in which two or more compounds must be present in order to elicit attraction.

Because *M. communis* is a significant pest on multiple crops, it was important to determine if the pheromone could be used as a tool for management and monitoring of this species. Therefore, we also determined the optimal dose of 13-tetradecenyl acetate to use in lures, and compared two types of release devices. The results from the dose experiments showed that the 10 mg dose of 13-tetradecenyl acetate resulted in the higher trap catches of *M. communis* males than lower doses. In the bioassays comparing lure devices, plastic sachet dispensers (that were used in other elaterid field bioassays) were compared to rubber septa dispensers, which are more commonly used as lures for many

pest species. There was minimal difference in the total numbers of males captured throughout the duration of the bioassays. However, traps baited with rubber septa appeared to capture male beetles more consistently throughout the bioassays than the sachets. This is most likely due to differences in the release characteristics of the two devices, with the septa having a lower but longer release rate than the sachets. However, this is speculation at this point because the release rates of the two devices were not measured. Overall, the results from this chapter should lead to the development of a commercial lure for this important pest species.

At the beginning of this dissertation, not much was known about the chemical ecology of North American elaterids. While going through the existing pheromone literature it became apparent that many of the pheromones that had been identified from European species (i.e. geranyl and neryl esters) were conserved among related taxa (reviewed in Tóth 2013; Tolasch et al. 2013; König et al. 2016). Therefore, we thought that we might be able to exploit this conservation of structures to identify possible pheromone components of related North American elaterids. Thus, we synthesized and field tested a panel of known elaterid pheromones.

Preliminary field screening trials of blends of these pheromones were carried out in southern California's San Bernardino National Forest. The neryl ester blend attracted males of *Idolus californicus* (subfamily Elaterinae). Neryl esters had previously been identified from European elaterids in the genera *Agriotes* (Tolasch et al. 2010), *Idolus* (Tolasch et al. 2013), and *Betarmon* (König et al. 2016). From GC-EAD bioassays, it was determined that antennae of males responded most strongly to neryl butyrate and neryl

hexanoate from amongst the neryl esters tested. However, field bioassay results showed that males were only attracted to neryl hexanoate, suggesting that it was likely to be a pheromone component for this species. Subsequent analyses of volatiles from crushed abdomens of females revealed that females produce neryl hexanoate and neryl octanoate, but not neryl butyrate. Results from field bioassays with the two pheromone candidates showed that males were only attracted to lures that contained the major component, neryl hexanoate, even though neryl octanoate also elicited antennal responses from males. The lack of attraction of *I. californicus* males to neryl butyrate and neryl octanoate in the field suggests that they may play a role in reproductive isolation among closely related species that share one or more neryl esters as pheromone components. The results from this study are the first to demonstrate conservation of pheromone structures between European and North American click beetles in the subfamily Elaterinae. In addition, this study also demonstrates proof of concept of the idea that there is conservation of pheromone structures between North American and European elaterids, and that field screening known elaterid pheromones can aid in the identification of pheromones for additional elaterids in North America.

During the field bioassays of the pheromone of *I. californicus*, males of a *Dalopius* species (subfamily Elaterinae) were captured in traps baited with neryl octanoate, the minor component identified from *I. californicus* females. Electrophysiological assays with a series of homologs showed that the antennae of *Dalopius* males only responded to this compound. These findings suggest that neryl octanoate is likely to be a pheromone component, and possibly the only component, for

this *Dalopius* species (subfamily Elaterinae). Unfortunately, we could not confirm that neryl octanoate is a sex pheromone component for this species because we were not able to collect female beetles to analyze their volatiles. Future research should follow up on this discovery to determine whether neryl octanoate is indeed produced by females of this *Dalopius* species, and whether they produce any additional pheromone components.

One continuing bottleneck in our research on click beetle pheromones is the difficulty in obtaining extracts that contain pheromone components for analysis. That is, work on most of the female-produced pheromones studied during this dissertation began by trying to collect headspace volatiles from individual females or groups of females held in glass jars. Analyses of the extracts showed that few females produced pheromone under these conditions (Table 2.1 in Chapter 2; Results in Chapter 4; unpub. data), due to a number of possible reasons (e.g. lack of proper lighting, insufficient space, lack of food, or other factors related to the unnatural conditions under which the beetles were held). In addition, all of the females used had been field collected, so most were probably already mated, and it is possible and even likely that females may stop producing pheromone after they are mated. One possible solution is to rear the beetles in the laboratory, so that newly emerged females can be harvested before they are mated, but this solution is not ideal because the life cycles of many of these species can be one to several years.

The pheromone glands of some female click beetles are located in the abdomen (Ivaschenko and Adamenko 1980; Merivee and Erm 1993), and most elaterid pheromone identifications have been done by extracting gland contents with solvent. However, for the species that I studied, I was unable to find the pheromone glands described in the

literature, despite carrying out dissections of females of several click beetle species. In addition, females of a *Cardiophorus* species from Europe also lacked the pheromone glands described above (T. Tolasch, pers. comm.), indicating that gland extracts are probably only useful for a subset of the Elateridae, because pheromone gland morphology clearly varies among species.

Because collections of headspace volatiles from live females were generally so unproductive, as a fallback, volatiles were collected from the headspace of crushed abdomens from freeze-killed females, using solid phase microextraction (SPME). This method was crucial in the identification of pheromones from the four click beetle species described in this dissertation. Even though this method was successful for some females, it suffers from the major disadvantage that it is essentially a sampling method only. That is, the volatiles collected are thermally desorbed directly into the GC or GCMS, so there is no material for bioassays or for derivatizations to help determine the structure.

The logical next steps of the work described here will be to screen all known elaterid pheromones and their analogs throughout the North America, and to work on pheromone identifications for click beetles outside of the subfamily Elaterinae. There are an estimated 1000 elaterid species in North America, and this dissertation describes the identification of sex pheromones and sex attractants for only five of those species, two in the subfamily Cardiophorinae and three in the subfamily Elaterinae. Thus far, it has been shown that two sympatric *Cardiophorus* species share a pheromone component, and that pheromones are conserved between European and North American *Idolus* species. In contrast, the sex pheromone components of *Melanotus communis* and its Japanese

congeners are considerably different, suggesting that they might be more distantly related than the previously mentioned *Idolus* and *Cardiophorus* species.

Because research into elaterid pheromones is in its infancy, we do not know how much widely pheromone structures are likely to be conserved within the family, the amount of variation to be expected in the pheromones across the family as a whole, and even if all pheromones will be female-produced sex pheromones. In addition, there is also a growing need to study the pheromones of pestiferous elaterids, so that they can be developed as tools for IPM programs. I hope that this thesis will serve as a foundation for further expansion of elaterid pheromone research in North America, both to develop a better knowledge of the chemical ecology of this large insect family, and to develop new tools to combat the growing wireworm problem in agricultural systems across the continent.

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