CHEMICAL ECOLOGY OF *IBALIA LEUCOSPOIDES ENSIGER*

by

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(Under the Direction of Kamal J.K. Gandhi)

ABSTRACT

Sirex noctilio F. is an invasive woodwasp native to Europe which has invaded countries in the Southern Hemisphere causing extensive damage to pine (*Pinus* spp.) stands. Its recent introduction into North America has generated concern for pine plantations in the southern United States. *Ibalia leucospoides ensiger* Norton (Hymenoptera: Ibaliidae) parasitizes the eggs and first instars of *S. noctilio*, and is considered one of its most effective biocontrol agents. My two research objectives were to: 1) identify and quantify the antennal sensillum of male and female *I. l. ensiger*; and 2) identify compounds that are attractive to *I. l. ensiger*. Antennae of *I l. ensiger* are sexually dimorphic, although there were no differences in the number of sensillum between sexes. Seven types of chemo- and mechano-receptors were identified on the antennae with varying numbers. In the laboratory, *Ibalia l. ensiger* showed oviposition responses to phytotoxic venom of *Sirex* spp. injected into logs and their host's oviposition holes. Using GC-EAD, 14 compounds from *Sirex* venom and oviposition holes were found to be electrophysiologically active with *I. l. ensiger* antenna.

INDEX WORDS: antennae, *Ibalia*, North America, parasitoid, *Sirex noctilio*

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DEDICATION

This thesis is dedicated to my family, friends, and to a patient major professor.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

THESIS INTRODUCTION

1.1 EXOTIC SPECIES

Exotic species cause major economic damage in their non-native habitats. They cause approximately $1 trillion losses worldwide annually, with ~$17 billion, and $120 billion losses occurring in Europe and United States respectively (Pimentel et al. 2005, Kettunen et al. 2008). Many exotic animal and plant species have been moved around the world either intentionally or inadvertently with consequences to non-native ecosystems. One classic example of an exotic species is lionfish (*Pterois miles* J.W. Bennett), an invasive species now established off the southern coast of the U.S. which was presumably first brought in through the aquarium trade (Semmens et al. 2004). Lionfish eat more than 40 different types of indigenous fish which in turn affects native trophic communities (Morris & Akins 2009). As of 2009, the estimated cost of managing the spread of lionfish was $1 million dollars over a 5 year period (Morris & Whitfield 2009). Water hyacinth [*Eichhornia crassipes* (Mart.) Solms] has become a major problem in China due to its ability to spread quickly. The plant invades shipping lanes hindering movement of resources as well as drainage and usage of water for irrigation (Penfound & Earle 1948, Zeiger 1962, Schmitz et al. 1993). The estimated cost of managing this invasive plant is $7 billion per year (Chu et al. 2006). Emerald ash borer (*Agrilus planipennis* Fairmaire) is an insect native to Asia and Eastern Russia. This pest

was discovered in Michigan in 2002, and is presumed to have been introduced in wood of shipping crates (solid wood packing material). About 53 million ash trees have been killed since its introduction with an associated cost of $10.7 billion over ten years (Kovacs et al. 2010).

In the U.S. alone, there are 50,000 exotic species that cause tremendous ecological damage across terrestrial and aquatic habitats (Pimentel et al. 2005). Among these species, exotic herbivorous insects contribute greatly to ecological changes at the ecosystem level (Gandhi and Herms 2010). Exotic herbivorous insects may cause defoliation and/or mortality of host trees. This results in greater canopy gap formations and coarse-woody debris, altered abundance of fauna, loss of food for indigenous species, and extinction of native species through direct or indirect interactions (Aslop & Laughlin 1991, Pimentel et al. 2005, Gandhi and Herms 2010). For example, gypsy moth (*Lymantria dispar* L.) has a long and well-documented history of impacting hardwood forests in the eastern U.S. This moth can feed on over 300 host species (Alalouni et al. 2013), and damages trees through defoliation. Repeated defoliation stresses the trees and predisposes them to colonization by additional insects and diseases and can ultimately results in tree mortality (Twery 1990).

There are three major stages in the invasion process by an exotic species: arrival, establishment, and spread (Liebhold et al. 1995). At each step of the invasion process, there are many abiotic and biotic factors that determine whether an exotic species becomes a successful invader (Niemelä and Mattson 1996). Abiotic factors include procedures for inspection of goods ports-of-entry; biotic factors include availability of native host species or otherwise suitable hosts. Arrivals of exotic species occur at various

ports of entry (seaports and airports) all the time (Haack 2006). Species that are hardy, have an affinity to human technology (synanthropogenic), and are cryptic and hide easily (e.g., in soil and wood) likely have the greatest arrival success. Species that are parthenogenic, can reproduce quickly, and can tolerate a wide range of environmental conditions, and have optimal hosts at their ports of entry can readily establish in the non-native habitats. Once the exotic species is established, then its dispersal ability and the density of suitable hosts determines its rate of spread across the landscape. For example, emerald ash borer likely arrived in Michigan as immatures in solid wood packing material (cryptic and resistant life-stages) and established on the abundant ash trees planted in urban areas adjacent to ports (presence of naïve host with little resistance mechanisms). Further, it can disperse easily by flight and has the capacity to locate suitable hosts even when ash is a minor component in the landscape.

1.2 EUROPEAN WOODWASP: *SIREX NOCTILIO* F.

In 2005, an exotic woodwasp, *Sirex noctilio* F. (Hymenoptera: Siricidae), a pest of conifer trees, was identified from pine monoterpene-baited funnel traps in Fulton County, New York (Hoebeke et al. 2005). Since that time, *S. noctilio* has been reported from Connecticut, Michigan, New Jersey, Ohio, Pennsylvania, and Vermont in the U.S., and Ontario and Quebec in Canada (NAPIS 2014). The native range of *S. noctilio* is Asia, Europe, and northern Africa but since the early 1900s it has spread to a number of other continents including Australia (including New Zealand and Tasmania), South Africa, and South America (Argentina, Brazil, Chile, and Uruguay) causing extensive economic to plantations of North American pine species. At its current spread rate of 30-50 km/year

spread it is predicted that in fifty years this insect may eventually inhabit forests from the Northwest passages in Canada to southern Florida, encompassing almost half of U.S. states (Haugen et al. 1990, Iede et al. 2012). There is a significant concern that *S. noctilio* may cause damage to pine (*Pinus* spp.) plantations that span millions of hectares in the southeastern U.S. with estimated costs of billions of dollars to local and regional economies (USDA-APHIS 2008).

Sirex noctilio colonizes primarily *Pinus*, although it occasionally reproduces in Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], true fir (*Abies* spp.), larch (*Larix* spp.), and spruce (*Picea* spp.) (Hopkins et al. 2008). Monterey pine (*P. radiata* L.), imported from California in the 19th century was a major host species for *S. noctilio* in Australia (Haugen et al. 1990). This woodwasp has been associated with pine species worldwide including *P. attenuata* Lemmon (knobcone pine), *P. banksiana* Lamb. (jack pine), *P. brutia* Ten. (Turkish pine), *P. canariensis* C. Sm. (Canary Island pine), *P. caribaea* Morelet (Caribbean pine), *P. contorta* Douglas ex. Louden (lodgepole pine), *P. densiflora* Siebold & Zucc. (Japanese red pine), *P. echinata* Mill. (shortleaf pine), *P. elliotii* Engelm. (slash pine), *P. halepensis* Mill. (Aleppo pine), *P. jeffreyi* Balf. (Jeffrey pine), *P. muricata* D. Don. (Bishop pine), *P. nigra* J. F. Arnold (European black pine), *P. palustris* Mill. (longleaf pine), *P. patula* Schiede ex Schltdl. & Cham. (patula pine), *P. pinaster* Arr. (maritime pine), *P. pinea* L. (Stone), *P. ponderosa* Douglas ex C. Lawson (ponderosa pine), *P. radiata, P. sylvestris* L. (Scots pine), and *P. taeda* L. (loblolly pine). Dinkins (2011) studied the host colonization and progeny development of *S. noctilio* in six North American pine species [eastern white (*P. strobus* L.) loblolly, longleaf, shortleaf, slash, and Virginia pines] and its native host (Scots pine) using bolts. The study

found that *S. noctilio* developed better on white, Virginia, and Scots pine bolts than other species, indicating that these three pine species may serve as suitable hosts in eastern U.S. (Dinkins 2011).

Sirex noctilio is considered a secondary pest in its native range as it is rare in forested stands (Hurley et al. 2007). However, it has caused significant ecological damage to pine plantations in the southern hemisphere where it has been introduced (Madden 1988, Haugen et al. 1990, Iede et al. 1998). It is noteworthy that all *Pinus* in the Southern Hemisphere are non-native and occur almost exclusively in monoculture plantations. Infestations of *S. noctilio* in such plantations cause losses in the millions of dollars through mortality to both vigorous and drought-stressed trees (Rawlings 1948). In contrast, mortality by *S. noctilio* in North America appears to be limited to pines that are overtopped or suppressed (Dodds et al. 2010). By attacking only weakened trees, it appears that *S. noctilio* in North America is acting as a secondary insect pest as in its native habitat (Dodds et al. 2010). Stand characteristics in North America such as the presence of many native pine species and a strong complex of competitors and biocontrol agents may be preventing outbreaks such as those in the Southern Hemisphere.

Sirex noctilio normally has one generation per year (Haugen 1990, Schiff et al. 2006). Males tend to emerge earlier than females, and they congregate in tree crowns waiting for emergent females to fly to these locations (Rawlings 1948, Morgan & Stewart 1966). After mating, oviposition generally takes place on the lower half of the tree (Morgan & Stewart 1966). The female will walk up the tree in a spiral fashion probing the bark surface with her antenna while dragging her ovipositor apparently assessing suitability of potential oviposition sites (Morgan 1966, Madden 1974). Once a location is

chosen, she will drill through the corky bark into the cambium layer and establish multiple tunnels. Females will deposit eggs in one tunnel and venom and a symbiotic fungus [*Amylostereum areolatum* (Chaillet ex Fr.) Boidin] into the others. The venom is phytotoxic and interferes with the translocation of water. As the tree cells desiccate, the symbiotic fungus begins to occupy the cellular space and spread. Once the eggs hatch, the larvae feed on wood/fungi. The larvae burrow deeper into the sapwood with successive instars (generally 5-12 depending upon temperature), and pupate just below the surface to emerge as adults (Neumann & Minko 1981). The combined effects of larval burrowing into wood, the symbiotic fungus, and phytotoxic venom overcome tree defenses and cause tree mortality.

Both silvicultural and biological control methods are used in the Southern Hemisphere for management of *S. noctilio* (Haugen 1990a, Hurley et al. 2007). Overcrowding of trees and lack of a thinning regime were aggravating factors for the initial *S. noctilio* outbreaks in Australia and New Zealand (Morgan 1966). Infested trees were removed and destroyed but this was considered too costly to be an effective strategy (Neumann et al. 1987, Haugen et al. 1990). Insecticidal sprays were tested on woodwasps, but these were ineffective in part due to the inaccessibility of the larvae deep inside wood (Rawlings & Wilson 1949). Thinning of stands to reduce basal area is the most effective strategy to prevent outbreaks of *S. noctilio* (Carnegie et al. 2003, Dodds et al. 2007, Hurley et al. 2007).

An entomophagic nematode, *Deladenus* (=Beddingia) *siricidicola* Bedding, discovered in 1962 was found to have approximately 50% parasitism rates in Australasia, ranges of <5-85% in South America, and up to 96% in Africa (Zondag 1969, Bedding &

Akhurst 1974, Hurley et al. 2007). This nematode does not affect the survivorship and oviposition rates of *S. noctilio* but renders the eggs sterile (Bedding et al. 1993), leading to reduction in populations of *S. noctilio* over time. Various species of hymenopteran parasitoids of woodwasps in both Europe and North America have been assessed for biological control potential in the Southern Hemisphere. *Rhyssa persuasoria* L. (Hymenoptera: Ichneumonidae) and *Ibalia leucospoides leucospoides* Hockworth (Hymenoptera: Ibaliidae) were imported into New Zealand in the early 1920-30's from England. *Megarhyssa nortoni nortoni* Cresson (Hymenoptera: Ichneumonidae) was imported along with *Ibalia leucospoides ensiger* Norton in the 1960's from North America, and *Rhyssa persuasoria himalayensis* Wilkinson was imported from India into New Zealand (Hurley et al. 2007). After a four-year study Morgan and Stewart (1966) concluded that there was a limited effect of *R. persuasoria* on *S. noctilio*. Results with *I. l. leucospoides* (and hybrids with *I. l. ensiger*) proved to be more efficient with parasitism rates averaging 30% but on occasion up to 55% (Hurley et al. 2007). The additive effect of *Rhyssa* spp. brought the parasitism rates of *S. noctilio* near 70% (Nutall 1989). Parasitoid releases for *S. noctilio* management in Australia began in the 1950's with *I. leucospoides*, *M. nortoni*, and *R. persuasoria* being the most effective parasitoid species (Hurley et al. 2007, Collett & Elms 2009).

1.3 STUDY ORGANISM: *IBALIA LEUCOSPOIDES ENSIGER* NORTON

Ibalia leucospoides (Hymenoptera: Ibaliidae) is a complex of two allopatric subspecies: *I. l. leucospoides* present in Europe, and *I. l. ensiger* in North America. Coyle and Gandhi (2012) reported that *I. l. ensiger* were found throughout the U.S. as well as in

all parts of Canada except Nunavut, New Brunswick, Newfoundland, Prince Edward Island, and Yukon territories. *Ibalia l. ensiger* is an endoparasitic koinobiont wasp native to the Nearctic region that parasitizes the egg or early first instars of woodwasps within *Sirex*, *Urocerus*, and *Xeris* (Smith & Schiff 2002). This species has been associated with the following tree genera: *Abies*, *Cupressus*, *Librocedrus*, *Picea*, *Pinus*, and *Tsuga* (Champlain 1922, Weld 1952, Cameron 1962, Yoshimoto 1970, Ryan & Hurley 2012).

Ibalia l. ensiger is a small wasp (~15 mm) with a black body and orange or rust colored, laterally flattened abdomen (Robertson & Gandhi 2013). In females, there is a knife-like appendage attached to the abdomen that sheathes the ovipositor which during oviposition extends perpendicularly to the body. Antennae are filiform with each sex having a different number of antennal segments, females with 13 and males 11 (not including the scape and pedicel). Males have a sinuous excavation on the first antennal segment. The appearance of the last antennal segment differs for females which have a more rounded tip compared to the more pointed tip for males. The last tergite on the abdomen is more triangular for the female and rectangular for the male.

This parasitoid species follows the life-cycle of its host and matures within one-year (Zondag 1959, Fernández-Arhex & Corley 2005). Females will locate the oviposition drill hole of *Sirex* spp., and apparently tests for suitability by inserting her antennae into the hole and palpating (Spradberry 1974). While her antennae are still inside the drill hole, she will slowly walk forward while simultaneously extending her oviposition sheath and finally inserting her ovipositor into the drill hole. The larva remains within the host until the third instar when it will exit the host and consume its remains before beginning pupation. Adults emerge in late summer, which generally

coincides with host emergence with males emerging slightly earlier than females (Spradberry 1974).The potential effectiveness of *I. l. ensiger* for biological control has been addressed in a number of rearing studies that determined the ratio of parasitoids to their siricid hosts in pine logs. Long et al. (2009) reared siricids and their parasitoids from logs in New York, and reported three species of parasitoids with approximately 21% *Sirex* spp. parasitism due to *I. l. ensiger*. Eager et al. (2011) had similar results for *I. l. ensiger* (16.4% parasitism rate) over a slightly larger region in New York. Barnes et al. (2014) reared native siricids from felled trees in three states (Georgia, Louisiana, and Virginia), and found up to 28% parasitism rates by *I. l. ensiger* on bolts in Louisiana. Hence, it seems that *I. l. ensiger* may exert considerable mortality on *S. noctilio* in its non-native habitat. However, its biological control potential may depend upon other factors e.g., population levels, competition from other parasitoids, local climatic conditions, and host quality across landscapes (Coyle and Gandhi 2012).

Several studies have shown that *I. l. ensiger* are attracted to the volatiles emanating from the symbiotic fungus, *A. areolatum*, which is inoculated with the *S. noctilio* egg in the tree. Spradberry (1974) extracted and cultured fungal spores from the mycangia of *S. noctilio* and observed the responses of *I. l. ensiger*. They concluded that volatiles from the fungus attracted the parasitoid to its host. Another experiment looked at the responses of the parasitoids to host oviposition densities, and found a correlation between host abundance and rate of parasitism (Fernández-Arhex & Corley 2005). More recently, Bryant (2010) assessed the specific compounds from the symbiotic fungi, and identified a number of compounds which stimulated ovipositor probing by *I. l. ensiger*.

However, they did not catch any *I. l. ensiger* using these compounds as trap lures in the field.

Considering that *I. l. ensiger* colonizes the egg and first instar of siricids, there may be host location cues that have been overlooked in previous studies. Since siricids primarily colonize stressed and damaged pines, a suite of monoterpenes (e.g., alpha-pinene, beta-pinene, and myrcene) generated as pine defensive responses and released upon damage may be acting as long-range attractants for *I. l. ensiger*. Further, siricids tend to inoculate the tree with relatively large quantities of the phytotoxic venom. This venom may be acting as a short-range cue to *I. l. ensiger* to find the precise location of the siricid oviposition hole. Our project goal was therefore to identify attractants for *I. l. ensiger* that might be employed in enhancing parasitoid activity in *Sirex*-infested stands, and trapping of *I. l. ensiger* in the field for mass-rearing or monitoring of parasitoid (and potentially host) population densities.

1.4 THESIS OBJECTIVES

The research objectives of this thesis were to: 1) identify and quantify antennal sensillum on both female and male antennae of *I. l. ensiger*. Chapter 2 identifies the types and numbers of sensillum present on antennal segments, and briefly addresses the likely function of identified sensillum with respect to insect behavior; 2) determine if there are specific host compounds associated with the *Sirex* venom and *Sirex* oviposition holes that may be used as chemical cues for host location by *I. l. ensiger*. Chapter 3 specifically tested the attraction of *I. l. ensiger* to three treatments (water, *Sirex* phytotoxic venom

from venom gland, and *Sirex* oviposition holes) over five time periods (0d, 4d, 8d, 12d, 16d) on pine logs.

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CHAPTER 2

ANTENNAL SENSILLA OF MALE AND FEMALE *IBALIA LEUCOSPOIDES ENSIGER* NORTON, A PARASITOID OF SIRICIDAE (HYMENOPTERA)[1]

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ABSTRACT

Over the past 50 years, the Eurasian woodwasp (*Sirex noctilio* F.), a pest of pine (*Pinus* spp.) trees, has been introduced into various non-native habitats in the Southern Hemisphere and North America. Some of the most effective biological control agents are hymenopteran parasitoids that parasitize various life stages of *S. noctilio*. Little is known about how these parasitoids locate their host in the wood, but presumably do so through chemolocation using antennae. We examined the external antennal morphology of *Ibalia leucospoides ensiger* Norton (Hymenoptera: Ibaliidae), a major parasitoid of siricids in North America. Males had two more antennal segments (13) than females (11) although the total antennal length was not significantly different. The first flagellomere of the male has a distinctive excavated region, which is absent in females. Using scanning electron microscopy (SEM), we identified seven types of antennal sensillum on male and female *I. l. ensiger* including campaniform, chaetica (types 1 and 3), coeloconic, fluted basiconica, placodea as well as pit-like organ of uncertain type. There were no differences in the total number of sensillum present on antennae of either male or female *I. l. ensiger*. However, females had greater numbers of chaetica type 1 and fluted basiconica, whereas males had more sensillum placodea. Sensillum were unevenly distributed along the antennae of both males and females. Chaetica type 1 were the most numerous sensillum followed by placodea, fluted basiconica, and the pit-like organ. We discuss the possible function of each of these sensillum and how they may mediate mate-location and oviposition by *I. l. ensiger*.

KEYWORDS

Campaniform, chaetica, coeloconic, fluted basiconica, *Ibalia leucospoides ensiger*, pit-like organ, placodea, Siricidae

2.1 INTRODUCTION

Sirex noctilio F. is a woodwasp native to Asia, Europe, and North Africa (Spradbery & Kirk 1978, Ryan & Hurley 2012). In its native habitat it is a secondary pest colonizing stressed pine (*Pinus* spp.) trees causing little ecological or economic damage (Hurley et al. 2007). Outside of this range, *S. noctilio* has caused economic losses in numerous countries in the Southern Hemisphere (Madden 1988, Haugen et al. 1990, Iede et al. 1998). Currently, *S. noctilio* is a pest in Argentina, Australia, Brazil, Chile, New Zealand, South Africa, and Uruguay and has recently (~2005) established in North America (Hoebeke et al. 2005, Pollard 2006, Hurley et al. 2007) .

Sirex noctilio is a woodboring insect in which the larvae mine lengthy gallery systems in the xylem of dead or dying hosts trees. They primarily colonize *Pinus*, although it has been reported in larch (*Larix* spp.) and spruce (*Picea* spp.) (Rawlings 1948). Woodwasps ovipositing into the xylem tissue of a host deposit a phytotoxic venom that prevents translocation of photosynthate and weakens or kills the tree (Coutts 1969). Further, ovipositing females deposit a symbiont fungus (*Amylostereum areolatum* Boidin) which grows readily in the newly-desiccated environment (Madden 1974). The larvae feed on the developing fungus while boring a gallery through the sapwood. The interactions between venom, fungus, and larvae along with numerous attacks on tree bole can result in tree mortality. Even if the process does not kill the tree, the resulting wood quality is diminished because of resinosis (natural plant defense mechanism) and the larval mines.

There have been hundreds of millions of dollars of economic losses due to *S. noctilio* worldwide (Rawlings 1948, Bedding & Akhurst 1974, Haugen 1990). *Sirex*

noctilio is a pest primarily in non-native pine plantations in the Southern Hemisphere. In these areas, *S. noctilio* colonizes typically stressed trees but at high densities will also colonize healthy trees (Haugen et al. 1990). Overstocking and drought increase risk of *S. noctilio* infestation (Rawlings 1948). In areas experiencing infestations, silvicultural treatments (particularly thinning) and biocontrol methods which utilize parasitic nematodes and parasitoids have been widely used to manage populations of *S. noctilio* (Hurley et al. 2007). The parasitic nematode (*Deladenus siricidicola* Bedding) renders the eggs of *S. noctilio* sterile without affecting their dispersal behavior; thus the nematode is able to spread in the population without human assistance (Bedding & Akhurst 1974). Several species of hymenopteran parasitoids in the genera *Ibalia*, *Megarhyssa*, and *Rhyssa* (Hymenoptera: Ibaliidae and Ichneumonidae) have been released in affected nations of the Southern Hemisphere with a parasitism rate of up to 60% parasitism (Hurley et al. 2007).

Among hymenopteran parasitoids, *I. l. leucospoides* Hochenworth from Eurasia and *I. l. ensiger* (Norton) from North America have been released in the Southern Hemisphere (Carnegie et al. 2005). The effectiveness of these parasitoids has varied within regions but parasitism rates commonly occur in the range of 20-40%. In Australasia, parasitism rates were as high as 55% but generally averaged 30% (Nuttall 1989). Similar rates are reported from South America. In Brazil, parasitism rates averaged 25% with highest reports of 40%, and in Argentina reported rates were 0-35%.(Eskiviski et al. 2004). *Sirex noctilio* was detected in Chile in 2001, and with the prior introduction of *Ibalia leucospoides* in 1997, parasitism rates ranged as 25-30% (Beeche et al. 2012).

It is remarkable that *I. l. ensiger* can efficiently find siricid hosts concealed deep within the wood of infested trees that are typically widely-scattered on the landscape. Presumably, the parasitoid may utilize both long-range, volatile chemical attractants to find siricid-infested trees and short-range cues to find siricid oviposition holes. Several studies have assessed means by which *Ibalia* locates *S. noctilio*, and these have primarily focused on volatiles apparently produced by the woodwasps' symbiotic fungi and released from the oviposition sites (Spradberry 1974, Fernandez-Arhex & Corley 2005, Bryant 2010). When female *S. noctilio* drills oviposition holes, they wound the tree causing the volatile components of resin and to be released from the site (Freeman & Beattie 2008). Such volatiles (dominated by mono- and sesquiterpenes) are known attractants to many different bark and woodboring insects including siricids (Costello et al. 2008). Currently a monoterpene based lure is in use for detecting populations of *S. noctilio*. Further, when *S. noctilio* deposits its egg into the drilled chamber, phytotoxic venom and fungal spores are injected into wood. Both the venom and the fungal inoculum may release volatiles that are uniquely associated with the location a suitable host for Ibalia. As the symbiotic fungus matures in the host it may also release compounds that have previously been isolated from cultures of these fungi and have been shown to be attractive to *I. leucospoides* in the laboratory (Madden 1968).

In insects the antennae are the major organs for sensing volatile chemical cues (Vinson 1976). Located on the antennae are various types of sensillum that receive airborne molecules and then transmit specific signals to the brain regarding the identity and concentrations of these compounds in the environment; this information guides the behavior of parasitoids and other insects (Hayes and Vinson 1971). Detection of

compounds specifically associated with the location of the host insect or its habitat allows the parasitoid to orient to its host. Insect sensillum fall within the functional classes of mechanoreceptor (touch), chemoreceptor (chemical), gustatory (taste), olfactory (volatile chemical), thermo-receptor (heat), and hygro-receptor (humidity) (Schneider 1964). Studies of types of sensillum present on the antennae and their distribution may shed light on the mechanisms by which a parasitoid may find a cryptic and widely-dispersed host.

Chrystal (1930) originally described the morphology of the *Ibalia* antennae. A more detailed analysis of the morphology was not possible due the limitations of microscopy at that time. Due to the seriousness of *S. noctilio* as a pine pest and the efficacy of *I. l. ensiger* as a biocontrol agent, it has become imperative to better understand the biology and ecology of this parasitoid species. Our two research objectives therefore, were to: 1) describe the external morphology of the antennae of *I. l. ensiger* using a scanning electron microscope (SEM); and 2) quantify the types and numbers of antennal sensillum present on male and female *I. l. ensiger*.

2.2 METHODS

2.2.1 Insect Collection

Adult *I. l. ensiger* were collected in late 2011 from forest stands in Grand Parish, Louisiana (N31°35’42.65 W92°25’.08). Live wasps were captured using intercept panel traps hung over log piles of loblolly pine (*P. taeda* L.) trees. These trees were cut in fall 2010 and stacked cross-wise with pine branches placed on top of logs to better attract siricids and their parasitoids (Barnes et al. 2014). Wasps from traps were collected every

2-3 days, and were sent with ice-packs via one-day shipping to the University of Georgia campus in Athens, Georgia. All wasps were then stored in 70% ethanol until use.

2.2.2 Scanning Electron Microscope (SEM) Preparation

Preserved *I. l. ensiger* specimens were placed in 2.5% ammonium hydroxide solution for one min, and a 0000 paintbrush was used to remove surface debris. Wasps were then washed in distilled water for one min and placed in 70% ethanol. The head and antenna were brushed again under higher magnification to obtain a debris free surface. Heads were excised and cleaned to remove any tissue residue.

Aluminum stubs were coated with black double-sided carbon tabs. Initial preparation involved excising both antenna and placing one with ventral side up and the other dorsal side up on opposite sides of the stub. After initial scanning, there were difficulties in capturing the sensillum within frame and subsequent specimens were placed with head intact upon Scanning Electron Microscope (SEM) aluminum stub with one antenna oriented perpendicular to the stub. This was to provide a continuous view of the surface of the antenna. Antennae were coated with a palladium/gold amalgam using a SPI Module Sputter Coater (Structure Probe, Inc., West Chester, Pennsylvania). Stubs were mounted on a modified stage with a 45° attachment. After 90 sec the stub was rotated 120° and recoated to ensure all sides of each antenna were adequately coated. Antennae were coated the same day they were prepared to minimize drying effects. A total of nine female and five male wasps were used in the study; fewer males were used since the traps primarily caught female wasps.

Images were taken with a Zeiss 1450EP variable pressure SEM (Carl Zeiss MicroImaging, Inc., Thornwood, New York). Four sequential images were taken of each antennomere including one dorsal, one ventral and two lateral views. The antennomere length was measured in each of the four images, and then averaged to obtain a single length per antennal segment. Average length was taken due to the antenna bending while in the SEM chamber. The four separate antennomere images were opened in Photoshop® (2011) and organized to allow a 360° view of the antennomere. This single image was then aligned via sensillum to eliminate any possibility of duplicate counting. Using Photoshop®, categories were assigned to each type of sensillum and then counted using the counting tool. Our terminology for sensillum morphology is that defined in Amornsak (1998), Bleeker (2004), Crook (2008), Onagbola (2008), and Zacharuk (1980).

2.2.3 Statistical Analyses

Statistical analyses were performed using SAS (2013). Each adult *I. l. ensiger* was taken as a unit of replication. Data were first checked for the assumptions of normality (Shapiro-Wilks test) and constant variance. Since data showed non-normality that could be not rectified by transformations, non-parametric tests (e.g., Mann Whitney U and Kruskall Wallis tests) were used for analyses. Tests were performed to determine differences in: 1) total numbers of sensillum (irrespective of types) between male and female *I. l. ensiger*; 2) total numbers of each sensillum type between males and females; and 3) total numbers of seven sensillum-types on the antennae, irrespective of sex.

2.4 RESULTS

2.4.1 Morphology of Antennae

Antennae of *I. l. ensiger* were filiform consisting of a scape, pedicel and a variable number of flagellomeres depending upon the sex of the insect (Fig. 2.1 A-C). Males had 13 and females had 11 flagellomeres (Fig. 2.1 A). The proximal flagellomere of the male had an excavated region composing roughly half of the overall segment area (Fig. 2.2.).

The mean ($\pm$ SE) length of antennae of males was 7.6 $\pm$ 1.1 mm and for females was 6.6 $\pm$ 0.7 mm. Although the number of flagellomeres varied between sexes, their antennae did not differ significantly in length ($P = 0.116$). Male flagellomeres were longest at the proximal end and decreased in length toward the distal end. The females had the same pattern except that the distal flagellomere was greater in length than the preceding four segments. The distal flagellomeres for both sexes were cylindrical at their base; however, they tapered to a point in males but were blunted in females.

2.4.2 Antennal Sensillum Types

Many studies have addressed the inconsistencies and lack of standardization of classification of insect antenna sensillum (e.g., Zacharuk 1980, Amornsak et al. 1998, Onagbola 2008). We identified seven types of antennal sensillum on *I. l. ensiger* based upon the descriptions of Schneider (1964), Norton and Vinson (1974), Amornsak (1998), Crook et al. (2008), and Onagbola (2008). The antennal sensillum found on *I. l. ensiger* included campaniform, chaetica (types 1 and 3), coeloconica, fluted basiconica, pit-like, and placodea.

Campaniform sensillum (Ca) were disc-shaped with a small peg situated in the center (Fig. 2.3 A). They were mostly absent on the scape, pedicel, and first flagellomere, but were present on the other segments (Tables 2.1 and 2.2).

Chaetica type I sensillum (ChSI) were thin and long with longitudinal ridges along the entire length and ended in a point (Fig. 2.3 B, C). The base of the sensillum was situated deep in a socket with no visible membrane. The length of ChSI varied along the antennae with the longest ones on the proximal segments and becoming shorter toward the distal segments. These sensillum were found over the entirety of the surface on all antennal segments of *I. l. ensiger* (Tables 2.1 and 2.2).

Böhm bristles or Chaetica type III sensillum (ChSIII) were shorter and more smooth compared to the Chaetica type I sensillum which were longer and striated (Fig. 2.1C). ChSIII were located primarily at the base of the pedicel (i.e., the juncture with the scape) in both males and females, and were absent on the other flagellomeres (Tables 2.1 and 2.2).

Sensillum coeloconica (Co) were deep pits with recessed, pointed cones. The opening to the pit was approximately 3µm in diameter (Fig. 2.4). Longitudinal grooves were present along the pointed cone portion of the sensillum. Co sensillum were mostly absent on the scape, pedicel, and first flagellomere, but were present on most of the other segments (Tables 2.1 and 2.2).

Fluted sensillum basiconica (FB) resembled ChSI but were thicker and formed a greater angle with the surface of the flagellomeres. The tips were blunt, and at the base there was a ring of wrinkled cuticle (Fig. 2.3 B). FB sensillum were present all over the

antennomeres except for the scape, pedicel, and first flagellomere of males, and the scape and pedicel of females (Tables 2.1 and 2.2).

Placoid sensillum (PS) were elongated plate-like organs along the longitudinal axis of the flagellomeres (Fig. 2.3 A). These sensillum were present on all segments except the scape, pedicel, and first flagellomere of both sexes. In females, there was a clear pattern where the numbers of PS increased from the proximal to distal flagellomeres. This trend was not seen in males (Tables 2.1 and 2.2).

Pit-like organs (PLO) were small holes or pits along the surface of the flagellomeres (Fig. 2.3 D). The distribution of these sensillum varied between the sexes as on females they were found on almost all of the antennal segments. In contrast, on males PLO were found only on the five proximal segments (Tables 2.1 and 2.2).

There were no differences in the total number of sensillum present on antennae between male and female *I. l. ensiger* (X^2 = 2.778; *d.f.* = 1, P = 0.096). For each sensillum type, the following trends were observed: 1) there were greater numbers of ChSI (X^2 = 7.471; *d.f.* = 1., P = 0.006) and FB (X^2 = 9; *d.f.* = 1., P = 0.003) sensillum present in females than males; 2) greater numbers of PS (X^2 = 9; *d.f.* = 1., P = 0.003) were present in males than females; and 3) there were no significant differences for ChSIII (P = 0.688), Ca (P = 0.061) and Co (P = 0.253) numbers between males and females.

Overall, the numbers of sensillum present on antennae were different among sensillum type (X^2 = 88.814; *d.f.* = 6, P < 0.001). ChSI were the most numerous sensillum followed by PS, FB, and PLO with non-overlapping standard errors (Fig. 2.5 A, B). Ca, ChsIII, and Co sensillum were quite rare on both male and female antennae (Fig. 2.5 B).

2.5 DISCUSSION

Ibalia l. ensiger searches for its host by climbing up the side of a tree and probing the surface with the underside of its antenna (Chrystal 1930, Martinez et al. 2006). Once it has located a site of interest (normally a *Sirex* oviposition site) it will insert its antenna into the hole and "feel" around presumably for cue(s) to determine if there is a suitable present Spradberry 1974, Bryant 2010). Chemical cues can be derived directly from the host, activities of the host, and the microenvironment of the host. For example, when *S. nigricornis* oviposits on a tree, she deposits an egg bathed in phytotoxic venom as well as symbiotic fungus to facilitate feeding once the egg hatches (Long et al. 2009). It is presumed that *I. l. ensiger* inserts their antennae into the oviposition site to ascertain if there are volatiles specific to the desired host. The act of inserting the antenna into the site implies the importance of chemosensory clues for *I. l. ensiger* in locating suitable hosts.

Conversely, oviposition by *S. nigricornis* itself may cause release of stress-associated volatiles from pine trees. *Ibalia l. ensiger* may detect these volatiles or defensive compounds emanating from the tree which signal that a suitable host may be present. Some parasitoids use their sensillum to test the host directly by brushing their antenna over the surface of the host or over the substrate in which host is present (Chrystal 1930, Doutt 1959).

Female *I. l. ensiger* use their antenna to find hosts but presumably not mates (as females have not been observed actively seeking mates), whereas males likely use their antenna for mate location but not host location. We found that although males had two more flagellomeres than females, there was no difference in total antennal length. The

distal flagellomeres in females were the longest; this flagellomere likely comes in direct contact with the oviposition drill hole or other host-associated substrate.

Interestingly, male and female *I. l. ensiger* shared all seven types of sensillum, but their numbers varied. Greater numbers of ChSI and FB sensillum were present in female *I. l. ensiger*, whereas greater numbers of PS were present in males. ChSI are considered mechanoreceptors and FB are used for physical contact with a substrate as likely occurs when female parasitoids investigate a *Sirex* oviposition site (Onagbola 2008). PS sensillum have been linked to mate and host habitat location, behaviors which would be expected for male *I. l. ensiger* (Wcislo 1995, Ochieng 2000, van Baaren 2007).

The most numerous sensillum on *I. l. ensiger* were ChSI. Chaetica sensillum are believed to be associated with either mechanoreception or contact chemoreception depending upon the pore structure of the sensillum (Altner and Prillinger 1980, Amornsak 1998, Van Baaren 2007, Crook 2008). Sensillum chaetica are sometimes identified as sensillum trichodea and this has led to confusion in sensillum classification (Schneider 1964, Zacharuk 1980, Amornsak 1998). Sensillum of this type have no pores and are situated deep within a socket which tapers to a point (Van Baaren 2007). Distribution of ChSI was even over the length of the antenna except for segment one of the male. Grooves were present on these sensillum, but our SEM was unable to achieve resolution sufficient determine if there were pores within these groves. Longer sensillum were found on segments with greater length and vice versa. Numbers of sensillum per unit length generally increased distally.

The next most abundant sensillum, PS, have been linked to mate and host habitat location for parasitoid wasps (Wcislo 1995, Ochieng 2000, van Baaren 2007). Basibuyuk

et al. (1999) studied the morphology of several cynipid wasps, including *Ibalia* species from the U.S., and produced a table which lists various properties associated with placoid sensillum. Twelve characteristics were used to classify sensillum differences including shape (elongate, elliptical or rounded), placement (elevated, at same level, or sunken), and internal structure (components of structure, septum, and position of aperature). This suggests that PS is highly morphologically variable among parasitoid species, and these varying functions may be associated with this morphology diversity.

CO and PLO sensillum are both pit-like sensillum and are generally similar in structure. However, the functions of CO and PLO differ depending upon the insect species and have been classified as chemo, thermo-hygro, or olfactory sensillum (Herzner 2004, Segura 2013). In our study, CO sensillum were deep pores with recessed, pointed cones. PLO sensillum also were pores, but are much smaller in size. It was not possible with the resolution of our SEM to see the pore interiors. Each of these sensillum types can have multiple subtypes. Snodgrass (1935) illustrates several types of CO sensillum and classifies them based upon the depth of the depression; they were called shallow CO sensillum “coeloconic” and deeper ones “ampullaceal”. However, classification of sensillum into subtypes is very difficult without transmission electron microscopy (TEM) or electrophysiological studies.

ChSIII are sensillum that resemble the larger ChS type I, but they are shorter and were found only on the scape and pedicel of *I. l. ensiger*. ChSIII are also known as “Böhm bristles.” These sensillum apparently function as proprioceptors that sense orientation of the associated appendage (Pringle 1938, Schneider 1964, Merivee 1999).

Considering that ChSIII were found only on scape and pedicel of *I. l. ensiger*, they likely provide information about the position of the antenna.

Ca sensillum are disc shaped with a small standing peg situated in the center and have been classified as either a mechanoreceptor (Pringle 1939, Moran 1971, Agren 1977, Klowden 2007) or a thermo- hygroreceptor (Suwannapong 2012). Confusion has arisen due to the placement and morphology of this sensillum. Ca sensillum were initially described by Hicks (1857) as having a dome-like structure, and in 1909 were named 'sensillum campaniformia' by A. Berlese (Moran 1971). The presence of a peg-like structure exiting from the center of the dome has led some to question if these sensillum were being classified correctly. More recently a new morphological class has been assigned (coelocapitular) and function attributed (thermo- hygroreceptor) to these sensillum (Yokohari 1982, Herzner 2004).

The perpendicular orientation of FB sensillum to the flagellomeres of *I. l. ensiger* suggests they may contact the surface of the substrate prior to other sensillum. Studies have suggested that the function of FB is contact chemoreception (Norton and Vinson 1974, Ochieng 2000, Onagbola 2008). In the parasitic wasp *Pteromalus puparum* L. (Hymenoptera: Pteromalidae) these sensillum have been named ST4-UP, uniporous trichodea type 4 (Dweck 2009). Isidoro et al. (1996) give a similar description for sensillum present on antennae of *Trissolcus basalis* Wollaston (Hymenoptera: Scelionidae), and they suggest that these sensillum are not only chemosensory in function but also mechanoreceptors.

2.6 CONCLUSIONS

The life history and observed behavior of each sex of *I. l. ensiger* imply the capacity of these insects to efficiently sense and respond to stimuli from outside sources. We found that antennae of *I. l. ensiger* are sexually dimorphic in numbers and structure of antennomeres. Seven types of antennal sensillum are present on both male and female *I. l. ensiger*: campaniform, chaetica (types 1 and 3), coeloconic, fluted basiconica, pit-like organ, and placodea. There were no differences between the sexes in the total number of sensillum present on the antennae. We did however find that female *I. l. ensiger* had greater numbers of sensillum chaetica type 1 and fluted basiconica, whereas males had more sensillum placodea. Chaetica type 1 were the most numerous sensillum followed by placodea, fluted basiconica, and pit-like organ which suggests the relative importance of these sensillum for host and/or mate location by *I. l. ensiger*.

2.7 ACKNOWLEDGEMENTS

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Table 2.1. Distribution and means ± SE of male *Ibalia leucospoides ensiger* antennal sensillum (n = 5).

Antennomere	CA[a]	ChSI[b]	ChSIII[c]	CO[d]	FB[e]	PS[f]	PLO[g]	Total
Scape	0 ± 0	248 ± 16	0 ± 0	0 ± 0	0 ± 0	0 ± 0	33 ± 3	281 ± 19
Pedicel	0 ± 0	88 ± 6	24 ± 4	0 ± 0	0 ± 0	0 ± 0	23 ± 2	135 ± 12
First	0 ± 0	441 ± 30	0 ± 0	0 ± 0	0 ± 0	0 ± 0	64 ± 5	505 ± 35
Second	3 ± 1	852 ± 54	0 ± 0	3 ± 1	8 ± 1	225 ± 26	5 ± 4	1096 ± 87
Third	2 ± 1	873 ± 63	0 ± 0	1 ± 0	10 ± 1	250 ± 29	2 ± 2	1138 ± 96
Fourth	1 ± 0	887 ± 60	0 ± 0	1 ± 0	10 ± 2	268 ± 31	0 ± 0	1167 ± 93
Fifth	1 ± 0	854 ± 31	0 ± 0	1 ± 0	12 ± 1	284 ± 31	0 ± 0	1152 ± 63
Sixth	1 ± 0	979 ± 63	0 ± 0	2 ± 0	11 ± 1	305 ± 28	0 ± 0	1298 ± 93
Seventh	0 ± 0	873 ± 62	0 ± 0	1 ± 0	9 ± 1	255 ± 25	0 ± 0	1138 ± 88
Eighth	1 ± 0	764 ± 37	0 ± 0	1 ± 0	11 ± 2	250 ± 25	0 ± 0	1027 ± 64
Ninth	1 ± 0	805 ± 57	0 ± 0	1 ± 0	11 ± 1	229 ± 23	0 ± 0	1047 ± 81
Tenth	1 ± 0	774 ± 23	0 ± 0	1 ± 0	12 ± 1	224 ± 22	0 ± 0	1012 ± 46
Eleventh	1 ± 0	750 ± 41	0 ± 0	1 ± 0	13 ± 1	203 ± 19	0 ± 0	968 ± 61
Twelfth	1 ± 0	690 ± 50	0 ± 0	1 ± 0	8 ± 1	180 ± 17	0 ± 0	880 ± 68
Thirteenth	1 ± 0	727 ± 61	0 ± 0	2 ± 0	14 ± 2	167 ± 16	0 ± 0	911 ± 79
Total	**14 ± 1**	**10605 ± 654**	**24 ± 4**	**16 ± 1**	**129 ± 15**	**2840 ± 292**	**127 ± 16**	**13755 ± 984**

[a]CA = Campaniform
[b]ChSI = Chaetica type 1
[c]ChSIII = Chaetica type 3
[d]CO = Coeloconica
[e]FB = Fluted Basiconica
[f]PLO = Pit-like organ
[g]PS = Placodea

Table 2.2. Distribution and means ± SE of female *Ibalia leucospoides ensiger* antennal sensillum (n = 9).

Antennomere	CA[a]	ChSI[b]	ChSIII[c]	CO[d]	FB[e]	PS[f]	PLO[g]	Total
Scape	0 ± 0	281 ± 26	0 ± 0	0 ± 0	0 ± 0	0 ± 0	31 ± 5	312 ± 31
Pedicel	0 ± 0	115 ± 8	24 ± 3	0 ± 0	0 ± 0	0 ± 0	28 ± 3	167 ± 14
First	0 ± 0	527 ± 32	0 ± 0	0 ± 0	2 ± 1	0 ± 0	27 ± 4	556 ± 37
Second	4 ± 1	1334 ± 100	0 ± 0	2 ± 0	17 ± 2	28 ± 3	9 ± 2	1394 ± 108
Third	2 ± 1	1456 ± 73	0 ± 0	2 ± 0	19 ± 4	27 ± 2	6 ± 2	1512 ± 82
Fourth	2 ± 0	1505 ± 101	0 ± 0	2 ± 0	23 ± 3	35 ± 4	3 ± 1	1570 ± 109
Fifth	1 ± 0	1487 ± 98	0 ± 0	1 ± 0	30 ± 5	52 ± 4	2 ± 1	1573 ± 108
Sixth	2 ± 0	1542 ± 105	0 ± 0	1 ± 0	39 ± 6	89 ± 6	2 ± 1	1675 ± 118
Seventh	1 ± 0	1213 ± 58	0 ± 0	1 ± 0	43 ± 4	112 ± 7	2 ± 1	1371 ± 70
Eighth	1 ± 0	1205 ± 74	0 ± 0	1 ± 0	50 ± 7	123 ± 9	1 ± 1	1381 ± 90
Ninth	1 ± 0	993 ± 59	0 ± 0	1 ± 0	41 ± 5	137 ± 9	1 ± 1	1174 ± 74
Tenth	1 ± 0	951 ± 57	0 ± 0	1 ± 0	39 ± 4	141 ± 8	0 ± 0	1133 ± 69
Eleventh	6 ± 1	1478 ± 106	0 ± 0	3 ± 1	63 ± 11	309 ± 18	0 ± 0	1859 ± 137
Total	**21 ± 3**	**14087 ± 897**	**24 ± 3**	**15 ± 1**	**366 ± 52**	**1053 ± 70**	**111 ± 21**	**15677 ± 1047**

[a]CA = Campaniform
[b]ChSI = Chaetica type 1
[c]ChSIII = Chaetica type 3
[d]CO = Coeloconica
[e]FB = Fluted Basiconica
[f]PLO = Pit-like organ
[g]PS = Placodea

FIGURE LEGEND

Fig. 2.1. Scanning electron image of (A) female (top) and male antennae of *Ibalia leucospoides ensiger*; (B) a frontal view of wasp's head with orientation of the antenna; and (C) a close-up view of scape (Sc), pedicel (P), and chaetica type 3 sensillum (ChSIII) on flagellomeres.

Fig. 2.2. 360° scanning electron image of the first antennal segment of *Ibalia leucospoides ensiger*.

Fig. 2.3. Different sensillum-types on flagellomeres of *Ibalia leucospoides ensiger* including (A) placoid (PS) and campaniform (Ca); (B) coeloconic (Co) and fluted basiconica (FB); (C) chaetica type 1 (ChSI); and (D) pit like organs (PLO) on the first flagellomere of male. Note the different scale on each image.

Fig. 2.4. (A) Coeloconica sensillum (Co) on the flagellomere of *Ibalia leucospoides ensiger*, and (B) its size and close-up view of the opening.

Fig. 2.5. Mean and standard error for the different types of sensillum on male (n = 5) and female (n = 9) *Ibalia leucospoides ensiger*.

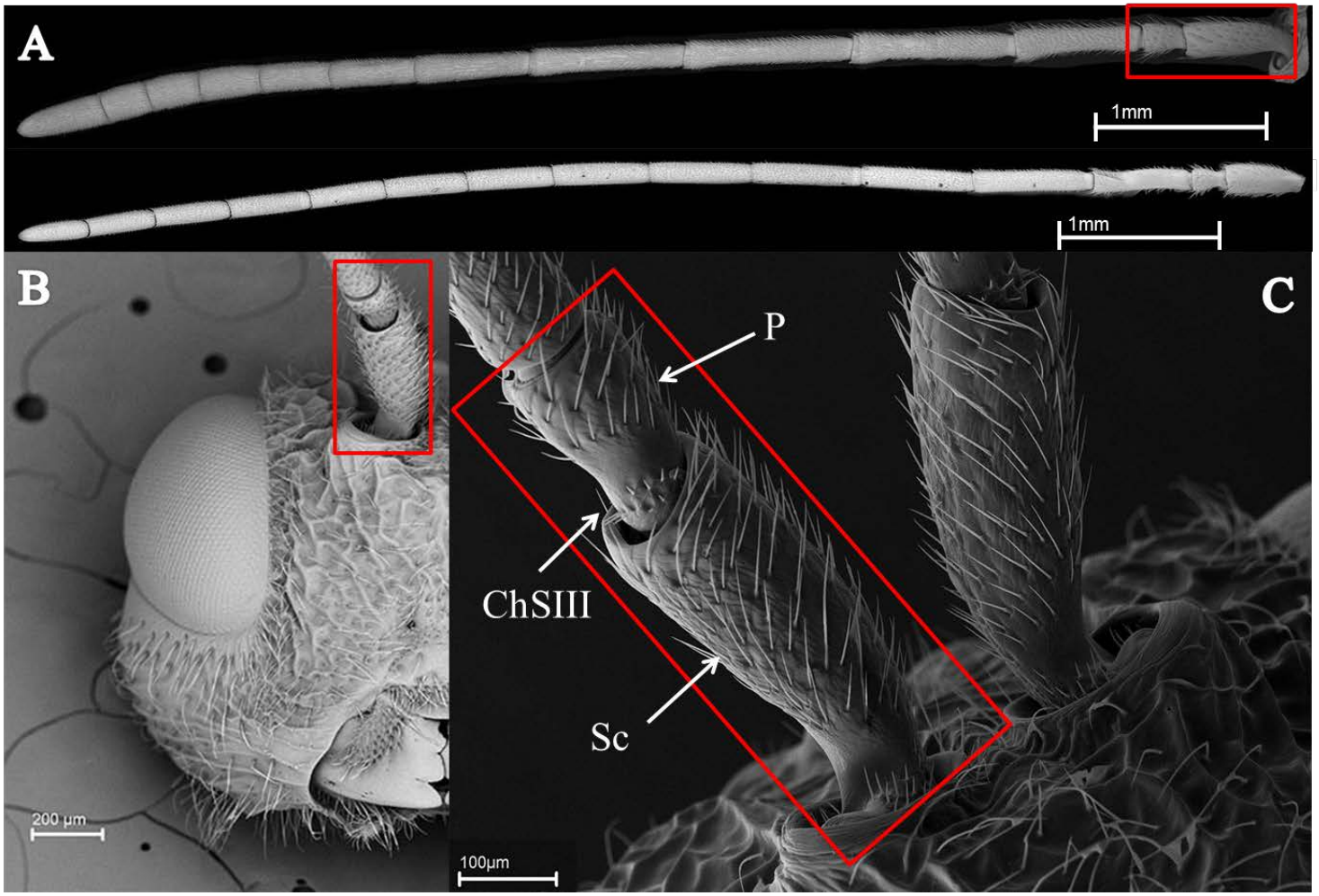

Fig. 2.1

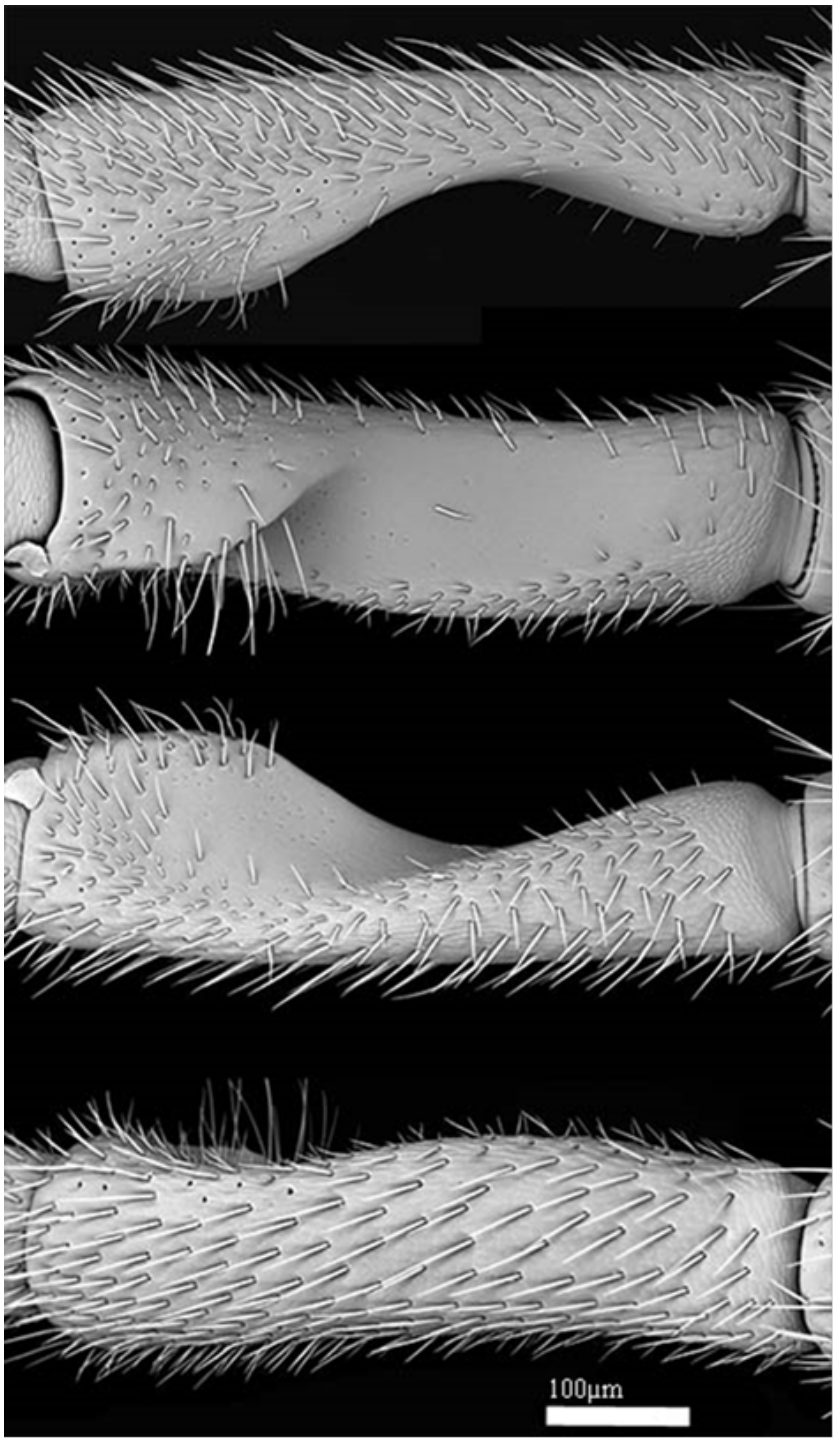

Fig. 2.2

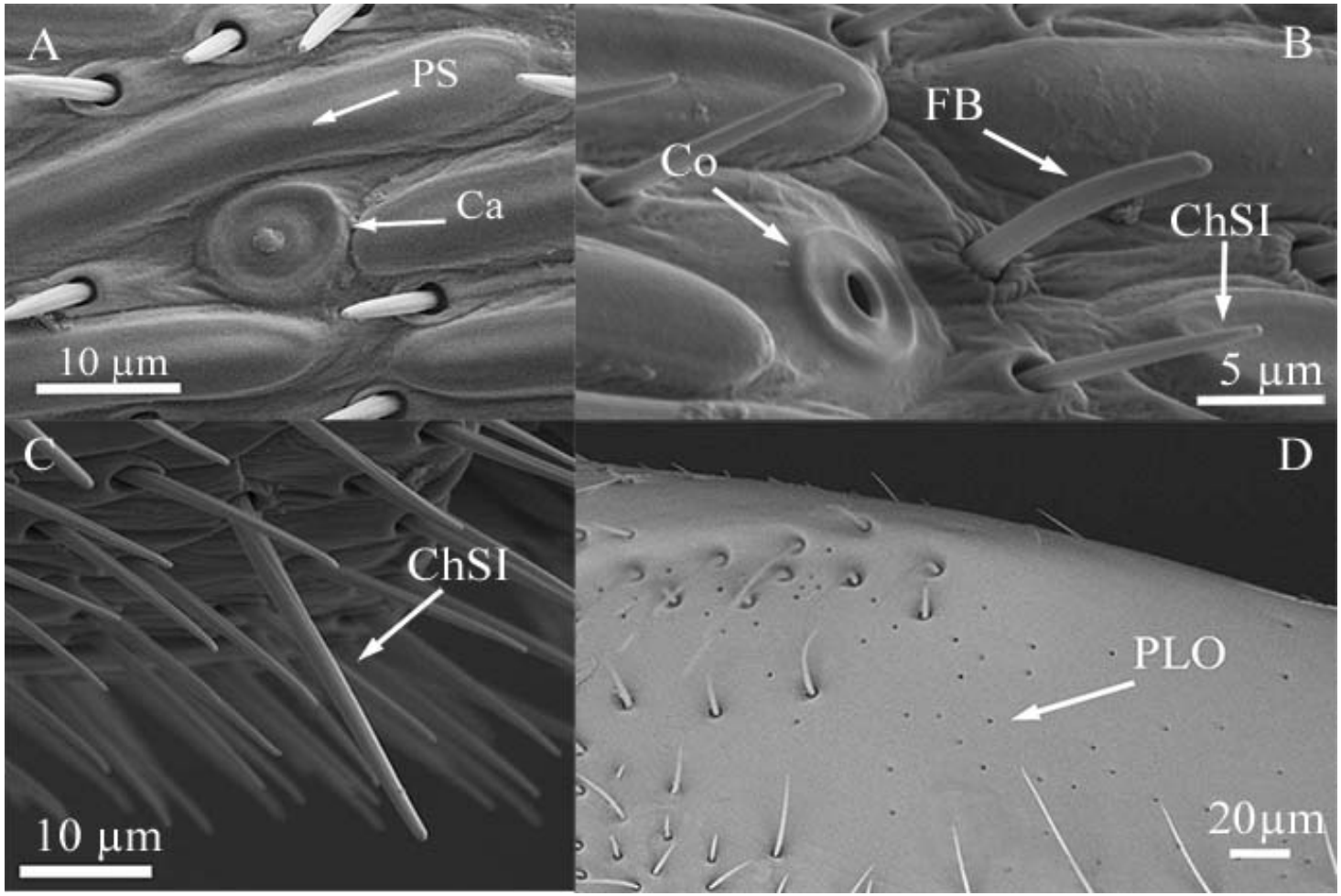

Fig. 2.3

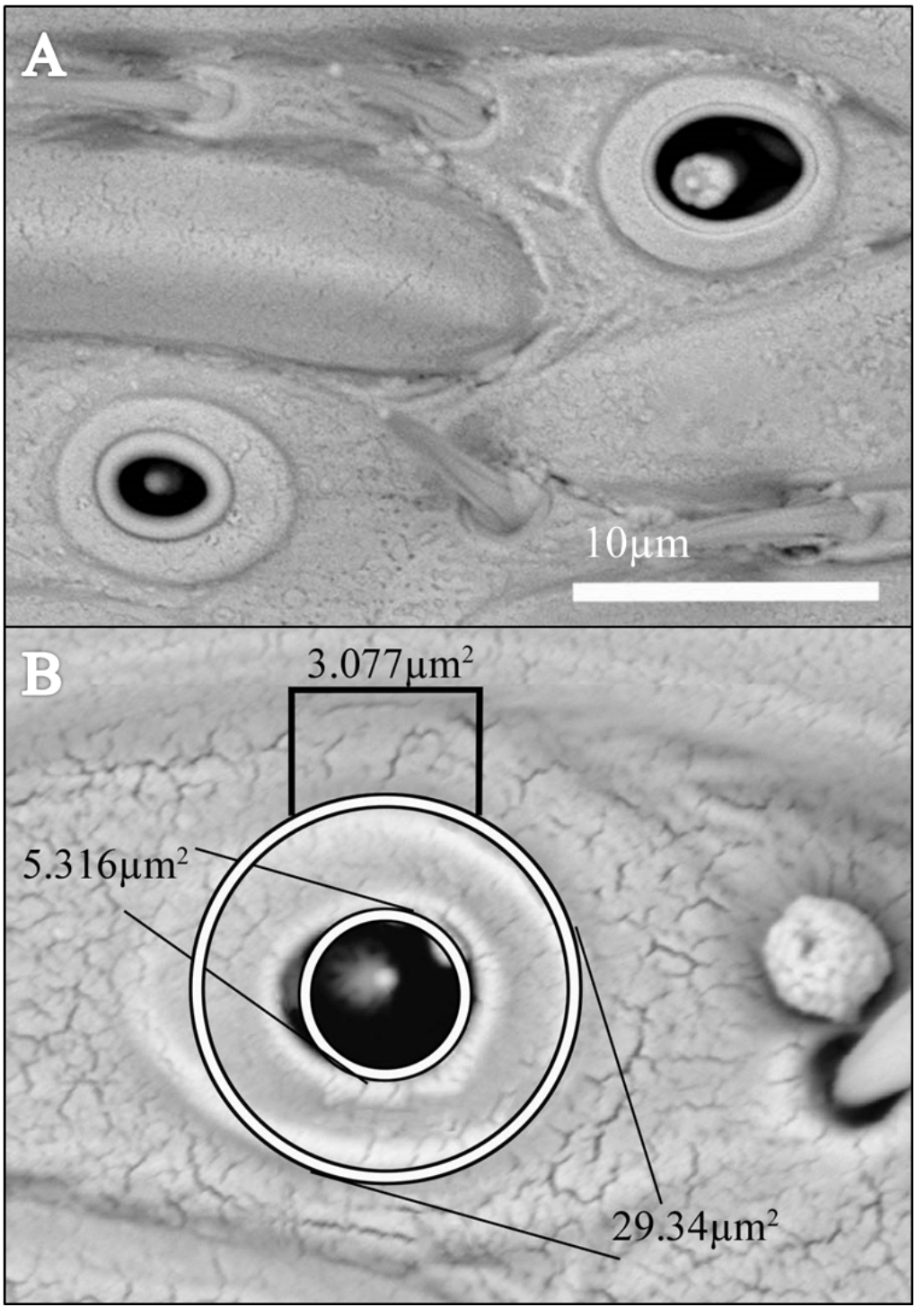

Fig. 2.4

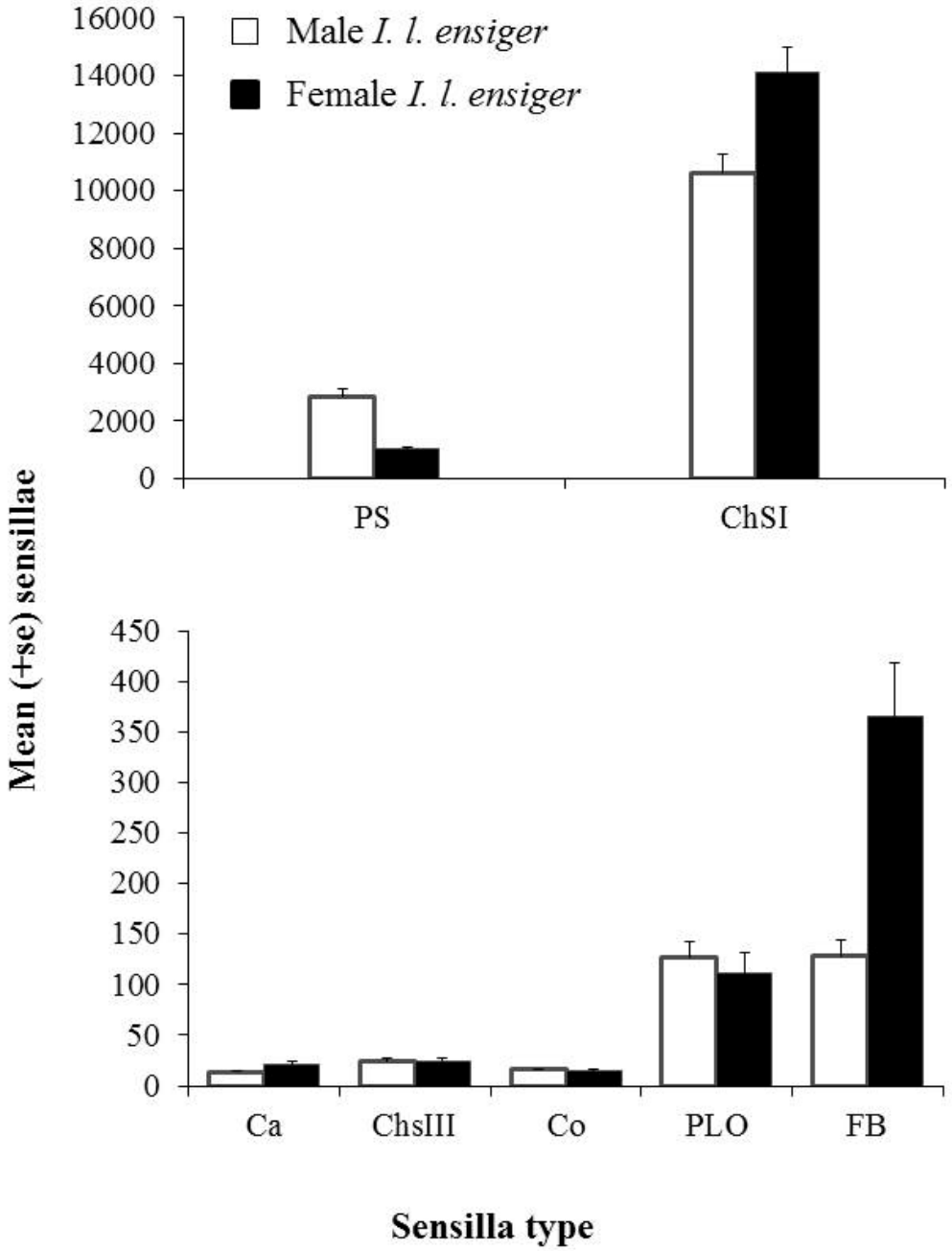
Male I. l. ensiger
Female I. l. ensiger
16000
14000
12000
10000
8000
6000
4000
2000
0
PS
ChSI
450
400
350
300
250
200
150
100
50
0
Ca
ChsIII
Co
PLO
FB
Mean (+se) sensillae
Sensilla type

Fig. 2.5

CHAPTER 3

RESPONSES OF *IBALIA LEUCOSPOIDES ENSIGER* NORTON TO HOST-ASSOCIATED VOLATILES

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[1]D. J. Robertson, B. T. Sullivan, W. P. Shepard, J. R. Meeker, C. W. Johnson, and K.J.K. Gandhi. To be submitted to Environmental Entomology.

ABSTRACT

Ibalia leucospoides ensiger Norton (Hymenoptera: Ibaliidae) is a native parasitoid of an exotic woodwasp *Sirex noctilio* and native siricids (Hymenoptera: Siricidae) on pine (*Pinus* spp.) trees in North America. As in many previously studied parasitoid-host systems, the ability of *I. l. ensiger* to locate its host likely depends upon specific chemical cues associated with the host itself or its location. Use of lures to attract this parasitoid to siricid-infested area could enhance siricid mortality and assist in capturing of parasitoids for mass-rearing purposes. This study tested responses of *I. l. ensiger* females to three treatments of pine logs [artificial oviposition drill holes injected with sterile water or *Sirex nigricornis* F. (Hymenoptera: Siricidae) phytotoxic venom, and *S. nigricornis* oviposition holes] over time (i.e., at 0, 4, 8, 12, and 16 days post-treatment). *Sirex nigricornis* was used as an indicator species in place of *Sirex noctilio*. Parasitism attempts by female *I. l. ensiger* were recorded, and volatiles from treatment sites on logs were analyzed using gas chromatography-mass spectrometry (GC-MS) and coupled gas chromatography electroantennographic detection (GC-EAD). Our results indicated that there were no parasitism attempts by *I. l. ensiger* to the water (control) treatment, few to venom only, and many to the oviposition holes of *Sirex* spp. on logs. Fourteen compounds were identified as eliciting electrophysiological responses in antennae of *I. l. ensiger*. When these compounds were presented at approximately equal concentration, the strongest responses by antenna of *I. l. ensiger* were to isopinocamphone followed by 4-allylanisole, *trans*-anethole, *trans*-pinocarveol, *trans*-verbenol, and verbenene. By day 16 of the experiment, the volatile profile of *Sirex* oviposition sites was distinct from that of *Sirex* venom-treated drill holes and water treated drill-holes. This oviposition site-associated blend can be produced synthetically and serve as a

prototype for an artificial lure to be assayed and refined in behavioral studies for attraction of *I. l. ensiger*.

KEYWORDS

Behavior, *Ibalia leucospoides ensiger*, GC-MS, GC-EAD, *Sirex* spp.

3.1 INTRODUCTION

One key to any organism's fitness is its ability to interpret information received through sensory inputs and adjust behaviors in ways which increase reproductive success. Doutt (1959) described four processes for a parasitoid to find a host: host habitat, host itself, host acceptance, and host suitability. When a female parasitoid first emerges from her pupation site, she determines her location, and in what direction she needs to travel to find a mate and host. Chemical, mechanical, and electromagnetic cues may steer the female in the right direction although random factors may be involved as well (Doutt 1959). Long range cues may lead to intermediary cues which pinpoint a location which has a suitable host. Once in the host's immediate vicinity, the parasitoid may use additional cues to identify the host itself or a suitable oviposition site.

Host location cues can arise from the host itself and its activities or the substrate where the host resides. For herbivorous insects, food plants may release defensive compounds to aid in warding off feeding; these compounds may be attractive to the insects' natural enemies such as parasitoids (Büchel et al. 2014). The amount of these compounds released is likely dependent upon the number of herbivorous insects feeding and size of host plant (Alborn et al. 1997). The movement of these compounds in forested areas is affected by the density of the stand and presence of mid- and understory plants, and the integrity and information value of the chemical signal can be affected by plume overlap with the same or different volatiles being produced by diverse plant species in the environment (Visser 1986).

Sirex noctilio F. (Hymenoptera: Siricidae) is a woodwasp native to Eurasia and North Africa (Spradberry & Kirk 1978). Outside of its native range, *S. noctilio* has caused significant damage to pine (*Pinus* spp.) plantations (Haugen 1990, Bain et al. 2012, Beeche et al. 2012,

Klasmer & Botto 2012 , Hurley et al. 2012, Iede at al. 2012). Damage to trees is caused by *S. noctilio* depositing eggs into the xylem tissue along with phytotoxic venom and a symbiotic fungus (*Amylostereum areolatum* Boidin). The phytotoxic venom interferes with the translocation of photosynthate which leads to necrosis of the surrounding tissue (Coutts 1969, Bordeaux & Dean 2012). The fungus then grows and colonizes the wood to provide the larvae a food source. The larvae of *S. noctilio* complete a number of larval instars while burrowing throughout the sapwood of the tree before forming a pupa near surface and finally emerging as an adult. The cumulative damage caused by the venom, fungal colonization, and larval mining inevitably leads to tree death (Madden 1981).

In its native range, *S. noctilio* is considered a secondary pest as it tends to attack dead or dying trees (Hall 1968). Outside of this range, *S. noctilio* is exposed to tree species that are naïve hosts often occurring in high-density plantations in regions of the world where pines are not native. In such areas, particularly in the Southern Hemisphere, *S. noctilio* causes significant mortality of apparently healthy trees (Morgan 1968, Ryan 2012). Since its detection in New Zealand in the early 1900s, *S. noctilio* has spread to South America, South Africa and more recently to North America (Ciesla 2003, de Groot et al. 2006, Dodds et al. 2007). In the northeastern United States where *S. noctilio* is currently established, it is primarily colonizing overtopped and suppressed pine trees (Dodds et al. 2010). The potential for *S. noctilio* becoming introduced or expanding its range into pine forests of the southern U.S. is considerable due to commerce along the eastern corridor, and the many, unthinned plantations of a susceptible host species (e.g., *Pinus taeda* L.) present in the southern region could provide optimal habitat for this exotic woodwasp.

Sirex spp. in North America have several natural enemies including an introduced entomophagous nematode and several species of hymenopteran parasitoids. In particular, *Ibalia leucospoides ensiger* Norton (Hymenoptera: Ibaliidae) is considered a major parasitoid of *Sirex* spp. Reports from the northeastern U.S. indicate that *I. l. ensiger* could have rates of up to 26% parasitism of *S. noctilio* (Long et al. 2009, Zylstra & Castro 2012). *Ibalia l. ensiger* is a koinobiont parasitoid which colonizes the egg and first instar of *Sirex* spp. where it remains until the third instar when it exits the host, consumes its remains, and pupates.

Studies have been conducted on the compounds which attract *I. l. ensiger* to its host. In these studies, odors from the fungal symbiont (*A. areolatum*) of *S. noctilio* were found to elicit attraction and apparent oviposition attempts by parasitoids (Coutts & Dolezal 1966, Madden 1968, Spradberry 1974, Martínez 2006). Research has since attempted to identify specific chemical compounds that are attractive to the parasitoids (Bryant 2010). Recently, Bryant (2010) identified several compounds isolated from *A. areolatum* cultures that elicited olfactory responses by *I. l. ensiger*. In addition to acetaldehyde which Madden (1968) identified as a behavioral stimulant, Bryant (2010) identified several other compounds which elicited behavioral responses in laboratory bioassays but not under field conditions.

The goals of this study were to identify host-related chemical cues that may be important for host location by *I. l. ensiger* seeking native siricid hosts in southern pine stands. For this study, *S. nigricornis* F. (native spp.) was used as a substitute for *S. noctilio*. Specifically, our research objectives were to: 1) assess variation over time in the behavioral responses of *I. l. ensiger* to natural *S. nigricornis* oviposition sites and artificial oviposition "drill holes" treated with siricid phytotoxic venom; and 2) identify compounds that may elicit these behavioral responses by assaying parasitoid olfactory sensitivity to compounds isolated from natural and

artificial oviposition sites and investigating correlations in the concentrations of these compounds to parasitoid behavioral responses in (1).

3.2 METHODS

3.2.1 Insect Collection

Live adults of *S. nigricornis* and *I. l. ensiger* were collected in fall 2012 by using intercept panel traps that contained various chemical lures (*alpha*-pinene, *beta*-pinene, ipsenol, ipsdienol, and fresh pine billets). Collections were made adjacent to a sawmill in Pineville, Louisiana (N31°35’42.65 W92°25’.08). In addition, ten loblolly pine (*P. taeda* L.) trees were cut in fall 2011, and stacked cross-wise at Whitehall forest in Athens, Georgia (N33°53'12 W83°21'42). In the summer of 2012, these logs were placed in rearing tents from which live *S. nigricornis* were collected every 2-3 days and shipped overnight to Pineville, Louisiana. Wasps were housed in clear screw-cap 120ml plastic containers (with puncture holes in lids for ventilation) with wetted kimwipe. *Ibalia* spp. were sexed with males being discarded. Initially five *Ibalia* wasps were placed into each container then later reduced to two. *Sirex* wasps were placed two to a container. Containers were placed into a Percival Scientific incubation chamber at 8°C with a 12L:12D light cycle.

3.2.2 Treatment Preparations

On 30 October, 2012 we cut 12 logs (1 m long and 15- 21 cm diameter) from two loblolly pine trees (six logs per tree) at the Homochitto forest in Mississippi (N 31°25'30 W 91°1'24) and immediately placed them into a walk-in refrigerator (4°C). Logs were removed on 31 October, 2012, and cut into 50 cm lengths using a Milwaukee Sawzall with an ethanol-

sterilized blade. Prior to initiating the experiment, one test log was selected at random to determine if logs were in a suitable condition for *S. nigricornis* oviposition. A single *S. nigricornis* was released on the log in an observation chamber and monitored. Once oviposition was observed, all logs were sprayed with ethanol to kill surface microbes (that might contaminate drill holes) and allowed to dry. Ends were sealed with Waxlor© sealant to slow the loss of moisture. Excess corky bark was removed to aid in locating oviposition sites following exposure to female *S. nigricornis*. Two sets (repetitions 1 and 2) of six logs were assigned numbers, and one log was selected at random for each treatment (water control, venom solution, or *Sirex* oviposition). This process was repeated for two additional loblolly pines on 25-26 November, 2012 for repetitions 3 and 4.

On 5 November, 2012, *S. nigricornis* females were released in an observation chamber (70cm x 50cm x 70cm; fig 3.1) onto a single log assigned to the *Sirex* oviposition treatment. As ovipositions were observed, oviposition sites were marked on the bark for future location and assigned serial numbers. Between 15 and 23 locations were tallied on each of the *Sirex* oviposition treatment logs. Locations of oviposition sites were traced onto a paper template which was then used to create identical spacing for the artificial oviposition drill holes on the *Sirex* venom and control treatment logs. This process was repeated on 7, 27, and 29 November, 2012 for the second, third, and fourth trees.

Artificial oviposition drill holes were made using a 1 mm diameter bit and Dremel® tool (Dremel®, Mount Prospect, IL) at locations defined by the template. The Dremel® tool was plugged into a Powerstat® (Superior Electric, Farmington, CT) to control motor speed and prevent the drill bit from burning the hole while drilling. The Dremel® was adjusted to 80 RPM, and bit was submerged in 95% ethanol and flame sterilized between treatment logs. Holes were

drilled on logs to a depth of 3 cm which is estimated depth of a live siricid oviposition site (Martinez 2006).

On 4 November, 2012, a venom solution was prepared from venom sacs excised from freshly killed female *S. nigricornis*. First, each female was dipped in ethanol to kill surface microbes and then dipped in sterilized distilled water to remove the ethanol. All dissection instruments used were submerged in 95% ethanol and flame sterilized between each insect. Five intact venom sacs were collected and each was placed into an autoclaved vial and weighed. An amount of sterilized distilled water equal to ten times the weight of the venom sac was added to each vial. The five vials were then placed into a cooler containing ice-packs, and agitated on a shaker table for 24 hours at 125 rpm to dissolve the contents of the venom sacks. At the end of the agitation, sack membranes were removed and the five vials were combined into a single stock of venom solution. About 20 µl of the ~9% venom solution was injected into each hole drilled into the designated venom log using a 10 µl syringe. Similarly, 20 µl of water was injected into the holes drilled into the control log using the previous method. This process was repeated again on 26 November, 2012 but ten venom sacs were used due to size variation. On 4 November, 2012 a sample of venom solution was plated on potato dextrose agar to check for possible fungal contamination during the process.

SPME (solid phase microextraction, Supelco Inc., Bellefonte, PA) fibers (2 cm long; adsorbent phase divinylbenzene/carboxen/polydimethylsiloxane), were used to collect volatiles from both natural and artificial drill sites. Volatiles were collected at four day intervals beginning on the day of inoculation (day 0). One end of a cylindrical headspace enclosure (approximately 0.6 ml volume; 6 mm diam. x 2 cm long) made of Teflon tubing was secured against the bark over the drill hole by a steel wire brace; the opposite end of the enclosure was sealed by

concentric, tightly-fitting pieces of Teflon tubing such that at their axis was a 0.8 mm opening that provided access to the interior of the headspace enclosure. The SPME fiber was inserted through this opening and exposed at room temperature to the headspace during a collection period of 30 min. Fibers were exposed at a distance of no more than 5 mm from the drill hole. Afterwards the tips of the SPME needles were sealed by inserting them into pieces of silicone GC septum (to prevent fiber contamination during storage) and were placed into screw cap culture tubes which were stored at -77° C until analysis (i.e., < 1 wk).

To estimate the contamination of the artificial and natural drill hole samples by background volatiles in the laboratory, three headspace collections were made of a clean glass surface placed in proximity (i.e., the approximate distance between logs on the lab bench) to the logs being sampled. Each collection was done by securing the headspace apparatus to a flat piece of glass cleaned with ethanol and dried. Fibers were exposed for a period of 30 minutes then analyzed using GC-MS identically as the other fibers.

3.2.3 Behavior of *Ibalia leucospoides ensiger*

In the observation chamber, a single treatment log was placed vertically on a lazy susan (to enhance observation of the entire log circumference) and a single female *I. l. ensiger* wasp was released at the bottom of the log and observed for 10 min. For each of the three treatment logs, five parasitoids were released one at a time. During the observation oviposition behavior of the parasitoid was recorded as binomial data (i.e., with 0 being no response and 1 an attempted oviposition). Parasitoids were used on a rotational basis with all exposed to the bioassay before reusing again. Toward the end of the repetitions 3 and 4, mortality of parasitoids necessitated usage more than repetitions 1 and2. These bioassays occurred the day after inoculation of

treatments into logs and every four days thereafter for a total of five bioassay periods (0, 4, 8, 12, and 16 days).

3.2.4 GC-MS and GC-EAD Analyses

Compounds collected onto the SPME fibers were identified and quantified relative to other treatments by GC-MS. Prior to analyses, SPME fibers were exposed at room temperature for 10 seconds in a septa-capped 0.25 l capacity amber glass bottle containing 10ml of a solution of an internal standard (C7Ac: Heptyl Acetate) dissolved 0.1 µl/ml in mineral oil. SPME fibers were inserted into a Hewlett-Packard 6890 gas chromatograph attached to a model 5973 mass-spectrometer. The column used was a Hewlett-Packard HP-5 (5% Phenyl Methyl Siloxane; 30m x 320 µm ID x .25 µm film thickness). Inlet temperature and carrier gas (helium) pressure was 240° C and 4 psi, respectively. Temperature program was 40° C for 0.7 min, 6° C/min to 180° C, 20° C/min to 240° C. Except where noted otherwise, peaks were identified by mass spectral matches to library spectra and retention time matches with identified standards when these were available.

GC-EAD analysis was performed on 27 female *I. l. ensi*ger for responses to volatiles sampled by SPME as described above. Thirteen *I. l. ensi*ger were tested using control samples (undamaged bark surface and water-treated drill holes) and 14 wasps were tested using natural oviposition sites of *S. nigricornis* over the range of sampling periods. The scape of an excised antenna was inserted into to a saline-filled $Ag/AgCl_2$ glass pipette reference electrode and the tip into an identical recording electrode which was attached to a high-impedance preamplifier and a data recorder. Beadle-Ephrussi ringers solution with 0.5% polyvinylpyrrolidone was used as the electrophysiological saline in the pipettes. Antennal preparations were placed in front of a 1 cm

diam. stainless steel tube that delivered a steady flow of charcoal filtered air (400 ml/min) into which eluent from the GC column was transmitted through a heated transfer line. The column and GC operating parameters used for GC-EAD were identical to those for the GC-MS analyses. Each antenna was used once per test. Coincidence in multiple runs of a GC peak at a particular retention time with an electrical response by the antenna was considered evidence of olfactory sensitivity to the compound eluting at that retention time.

Results from the GC-MS were not measured in units of concentration (e.g., ng/l) due to the inherent difficulties in calibrating SPME fibers in order to measure exact concentrations in sampled air. Thus measured quantities were scalar in nature and valid only for comparisons within each compound (i.e., different compounds could not be compared quantitatively to each other) for contrasts among treatments and time periods.

3.2.5 Statistical Analyses

Statistical analyses were performed using RStudio (2009-2013) and SAS (2013). Data were first checked for the assumptions of normality (Shapiro-Wilks test) and constant variance. Each pine tree used to obtain experimental logs was considered the unit of replication. To assess differences in binary responses (0 and 1) of female *I. l. ensiger* to treatments across the five time periods, a logistic regression was performed where Chi-square (X^2) values were calculated using the Wald test. Since *I. l. ensiger* did not respond at all to water inoculated logs, this treatment was removed from the analyses. Data for 14 compounds eluting from natural and artificial drill holes showed non-normality that could be not rectified by transformations. Hence, non-parametric tests (e.g., Mann Whitney U and Kruskal Wallis tests) were used to assess differences

among treatments and time periods where separate analyses were performed for these two factors.

To determine whether treatment-specific blends of volatiles were produced, we analyzed the proportions among the 14 olfactory stimulants using cluster analysis with Bray-Curtis similarity index (PC-ORD, version 6.0) (McCune et al. 2002). We analyzed only days 0 and 16 for each treatment since we were interested in trends at the limits of our sampling period (i.e., the times when *I. l. ensiger* exhibited the weakest and strongest, respectively, behavioral responses to treatments). Separate replicates for each of the treatments were included in cluster analyses. Based on the analysis results, dendrograms for all three treatments were created separately for 0 and 16 days.

3.3 RESULTS

3.3.1 Behavioral assay

Logistic regression indicated that both treatment (*Sirex* venom or natural oviposition holes) ($X^2 = 4.7$, *d.f.* = 1, $P = 0.03$) and time period (0, 2, 4, 8, 12, and 16 days) ($X^2 = 7.7$, *d.f.* = 1, $P = 0.006$) were significant factors affecting responses by female *I. l. ensiger* on treatment logs (Fig. 3.1). There was no response by female *I. l. ensiger* to the control treatment (water only), which was excluded from contrasts. Tukey tests indicated that there were significant differences between 0 and 16-day sampling-periods with no differences amongst other times (Fig. 3.1).

3.3.2 GC-EAD and GC-MS

GC-EAD followed by GC-MS analyses isolated and/or identified 15 peaks from *S. nigricornis* oviposition drill-holes which consistently produced antennal responses in female *I. l.*

ensiger (Fig 3.2, Table 3.2). The majority (eight) of these compounds were hydrocarbon monoterpenes (camphene, limonene, myrcene, *alpha*-pinene, *beta*-pinene, terpinolene, tricyclene, and verbenene); four were oxygenated monoterpenes (isopinocamphone, pinocamphone, *trans*-pinocarveol, and pinocarvone); two were phenylpropanoids (4-allylanisole, and *trans*-anethole), and one could not be identified. Standards were not available for retention time confirmations for verbenene and pinocarvone, so these identifications require confirmation. Fourteen compounds associated with insect infestation of pines (including nine of those identified as EAD-positive in analysis with SPME samples) were mixed in equal amounts (30-50 ng/µl), and tested by GC-EAD again to confirm olfactory sensitivity to specific compounds and compare relative responsiveness. In this test, the strongest EAD responses (deflection amplitudes) by female *I. l. ensiger* were to the following compounds: isopinocamphone, *trans*-anethole, verbenone, 4-allylanisole, *trans*-pinocarveol, and *trans*-verbenol (Fig. 3.3).

Kruskal Wallis tests indicated that there were significant differences for all 14 compounds (except for *alpha*-pinene and *beta*-pinene for time-period) among the three treatments and five time-periods (Tables 3.2, 3.3). The following general trends were observed for treatment effects: 1) greater amounts of 4-allylanisole, *alpha*-pinene, *beta*-pinene, camphene, limonene, terpinolene, and *trans*-anethole were produced by *Sirex* oviposition sites than the venom- or water-treated drill holes; and 2) greater amounts of isopinocamphone, myrcene, pinocamphone, and pinocarvone were produced by drill holes treated with *Sirex* venom than the other two treatments (Table 3.2). None of the compounds were present in greatest quantities from the water-treated drill holes. Furthermore, almost all of the compounds were produced in greatest amounts on days 0 or 4 after treatment establishment with diminishing amounts by 16 days (Table 3.3).

The dendrogram created from cluster analysis for day 0 of the experiment suggests that there were few or no differences in the blends of chemicals released by water- and venom-treated drill holes and natural *Sirex* oviposition holes (Fig. 3.4). However, the composition of the chemical blend produced from *Sirex* oviposition holes was distinct from that of the other two treatments by day 16 (Fig. 3.5).

3.4 DISCUSSION

Our study found that *I. l. ensiger* females made oviposition attempts in response to both *Sirex* oviposition sites and, to a lesser extent, drill holes treated with *Sirex* venom. There was no response by parasitoids to water/control treatment; this was expected as the literature has shown only host-specific compounds to produce insect attraction (Madden 1968). It seems that the host or host-released substances have to be present for oviposition attempts by *I. l. ensiger* to occur on pine trees. The response of *I. l. ensiger* to the venom treatment was surprising since studies of attractants of *Sirex* parasitoids have generally ignored possible odors produced by the venom itself or its effects on the host (and its effects on natural tree compounds) (Madden 1968) or the interaction between venom and the symbiotic fungus (*Amylostereum* spp.). Venom solution was plated on PDA to test for contamination during the extraction of the *S. nigricornis* venom sac, but this did not reveal any fungal contamination. However, this venom solution had been stored frozen prior to plating which could have affected viability of fungal propagules. Pure venom alters tree physiology through desiccation and necrosis possibly creating odor blends similar to those produced by a natural oviposition. Coutts (1966, 1967, 1969a, 1969b) found that the venom and fungus separately would not kill the tree, and that the two have to be present for eventual tree death to occur.

Cluster analysis results indicated that by day 16, there were major differences in the composition of chemicals arising from water- and venom-treated drill holes and *Sirex* oviposition sites. Furthermore, the responses of *I. l. ensiger* to the *Sirex* oviposition holes were greater in day 16 than day 0 of the experiments. Madden (1968) tested the behavioral responses by *I. l. ensiger* to *P. radiata* D. Don logs infested by *S. noctilio* and surmised that the attractants were volatiles associated with the fungal symbiont. Bryant (2010) identified several compounds from fungal symbionts (*A. areolatum* and *A. chailletii*) that elicited responses by *I. l. ensiger* in the laboratory but failed to yield results with field trials.

Greater amounts of 4-allylanisole, *alpha*-pinene, *beta*-pinene, camphene, limonene, terpinolene, and *trans*-anethole were present in *Sirex* oviposition sites than the artificial treatments. However, relatively greater amounts of isopinocamphone, myrcene, pinocamphone, and pinocarvone were found in *Sirex* venom-treated drill holes. Interestingly, when the antennae of *I. l. ensiger* were tested with equal amounts of 14 compounds associated with insect-infested pine boles, strong EAD response amplitudes were produced by the oxygenated monoterpenes isopinocamphone, *trans*-pinocarveol, *trans*-verbenol, and verbenone. Similarly, disproportionate sensitivity to oxygenated monoterpenes has been found for hymenopteran parasitoids attacking bark beetles [e.g., *Roptrocerus* spp. attacking *Ips grandicollis* Eichhoff in North America (Sullivan et al. 2000) and *I. typographus* L. in Europe (Pettersson 2001), and *Coeloides bostrichorum* Giraud attacking *I. typographus* in Europe (Pettersson et al. 2001)]. Oxygenated monoterpenes are present when pine trees are stressed and may also arise due to the action of microorganisms and insects in trees (Leufvén et al. 1988, Pettersson et al. 2001). Hence, these compounds may be providing general signals to *I. l. ensiger* that the tree is infested with bole-feeding insects and associated fungi and thus that siricids might potentially be present.

Overall, we conclude that *I. l. ensiger* is highly cued in to *Sirex* oviposition sites on pine logs. It is likely that monoterpenes such as *alpha-* and *beta*-pinene may be acting as long-range attractants but oxygenated monoterpenes such as isopinocamphone and pinocarveol may be acting as shorter-range attractants. Future testing of these compounds in laboratory bioassays is needed, and if some compounds are found to be behaviorally active with *I. l. ensiger*, then they could be tested under field conditions. It would be interesting to conduct similar experiments on other parasitoid species in the genera *Rhyssa* and *Megarhyssa* that tend to parasitize the later instars and pupal stages of siricids (Coyle & Gandhi 2010). Results from this study could be used to refine trapping methods for *I. l. ensiger* in southern pine stands when and if *S. noctilio* arrives in this region.

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Table 3.1. Weights of venom sacs (gm) and amounts of distilled water added to each sample to create a 1% venom solution for inoculation into pine logs.

Sample	Experiment 1/2 (prepared 4 Nov., 2012)		Experiment 3/4 (prepared 26 Nov., 2012)		Experiment 3/4 (prepared 28 Nov., 2012)	
	Sac Wt. (g)	**Water**	**Sac Wt. (g)**	**Water**	**Sac Wt. (g)**	**Water**
1	0.01546	0.15599	0.00732	0.07320	0.01245	0.12195
2	0.05270	0.50720	0.01023	0.10646	0.00909	0.09112
3	0.01963	0.21699	0.01017	0.11212	0.00852	0.08943
4	0.02050	0.22042	0.01400	0.14082	0.00170	0.03008
5	0.04224	0.40170	0.01663	0.16937	0.01362	0.14375

Table 3.2. Means (± SE) amounts of compound collected from either natural drill-holes of ovipositing Sirex or artificial drill-holes treated with either water or Sirex venom. Chi-square values are for each compound over the five time periods.

Compound	**Treatment**	**Amounts (Mean ± SE)**	**Statistical values**
4-Allylanisole	Water	4277.7 ± 941	
	Sirex Oviposition Holes	11349.3 ± 5703	$X^2 = 8.7$, *d.f.* = 2, *P* = 0.013
	Sirex Venom	7625.4 ± 3066.4	
alpha-Pinene	Water	1251552.8 ± 307046.6	
	Sirex Oviposition Holes	2514481.5 ± 1744562.2	$X^2 = 7.8$, *d.f.* = 2, *P* = 0.021
	Sirex Venom	715644.6 ± 166428.5	
beta-Pinene	Water	792964.2 ± 179140.5	
	Sirex Oviposition Holes	997028.4 ± 575914.1	$X^2 = 8.8$, *d.f.* = 2, *P* = 0.012
	Sirex Venom	419406.8 ± 87774.9	
Camphene	Water	81020.7 ± 15522.4	
	Sirex Oviposition Holes	95907.8 ± 40479.5	$X^2 = 9.1$, *d.f.* = 2, *P* = 0.010
	Sirex Venom	91949.2 ± 19648.5	
Isopinocamphone	Water	2839.8 ± 1243.3	
	Sirex Oviposition Holes	1802.1 ± 649.9	$X^2 = 5.2$, *d.f.* = 2, *P* = 0.076
	Sirex Venom	3272.1 ± 1206.2	
Limonene	Water	431532.8 ± 100377.8	
	Sirex Oviposition Holes	874033.2 ± 526973.1	$X^2 = 11.0$, *d.f.* = 2, *P* = 0.004
	Sirex Venom	391633.6 ± 128207.1	
Myrcene	Water	182171.5 ± 64524.3	
	Sirex Oviposition Holes	179109 ± 67834.3	$X^2 = 16.0$, *d.f.* = 2, *P* < 0.001
	Sirex Venom	219837.4 ± 101377.4	
Pinocamphone	Water	3331 ± 778.2	
	Sirex Oviposition Holes	2541.1 ± 543.4	$X^2 = 9.5$, *d.f.* = 2, *P* = 0.009
	Sirex Venom	4487.4 ± 1000.9	
Pinocarvone	Water	1064.3 ± 234.9	$X^2 = 8.5$, *d.f.* = 2, *P* = 0.014

	Sirex Oviposition Holes	1039.2 ± 249.1	
	Sirex Venom	1556 ± 362.5	
Terpinolene	Water	14506.6 ± 3591.5	
	Sirex Oviposition Holes	28035.6 ± 11378.3	$X^2 = 6.9$, *d.f.* = 2, *P* = 0.032
	Sirex Venom	23945.4 ± 7817.6	
trans-Anethole	Water	4277.7 ± 941	
	Sirex Oviposition Holes	11349.3 ± 5703	$X^2 = 8.6$, *d.f.* = 2, *P* = 0.014
	Sirex Venom	7625.4 ± 3066.4	
trans-Pinocarveol	Water	2726.1 ± 543.2	
	Sirex Oviposition Holes	3737.3 ± 1690.9	$X^2 = 9.1$, *d.f.* = 2, *P* = 0.011
	Sirex Venom	3016 ± 555.4	
Tricyclene	Water	12265.2 ± 2446	
	Sirex Oviposition Holes	14950 ± 7312	$X^2 = 10.1$, *d.f.* = 2, *P* = 0.006
	Sirex Venom	16718.3 ± 3892.3	
Verbenene	Water	9670.4 ± 1519.8	
	Sirex Oviposition Holes	8663.8 ± 2031.9	$X^2 = 10.6$, *d.f.* = 2, *P* = 0.005
	Sirex Venom	13353 ± 2756.7	

Table 3.3. Mean (± SE) amounts of 14 compounds eluted from artificial and natural drill-holes in pine logs over time. Chi-square value is for each compound over three treatments.

Compound	Sampling Period (days)	Amounts (Mean ± SE)	Statistical values
4-Allylanisole	0	385833.2 ± 136701.1	
	4	556457.3 ± 307035.2	
	8	90985.2 ± 29147.8	X^2 = 29.6274, *d.f.* = 4, *P* < .0001
	12	118980.2 ± 40363.2	
	16	72084.4 ± 20570	
alpha-Pinene	0	1282735.2 ± 364596.9	
	4	3881047.8 ± 3395745.6	
	8	1255561.9 ± 442266.2	X^2 = 2.7686, *d.f.* = 4, *P* = 0.5973
	12	699442.6 ± 208270.2	
	16	794411.4 ± 269436.7	
beta-Pinene	0	746089.3 ± 158321.1	
	4	1411307 ± 1117137.1	
	8	657313.2 ± 228888.6	X^2 = 28.8438, *d.f.* = 4, *P* < 0.0001
	12	457885.6 ± 126234.7	
	16	523025.3 ± 168455.4	
Camphene	0	106815.5 ± 21627.8	
	4	175422.4 ± 78485	
	8	55525.7 ± 16204.8	X^2 = 30.7649, *d.f.* = 4, *P* <0.0001
	12	55367.9 ± 22123.6	
	16	57729.3 ± 13552.7	
Isopinocamphone	0	3391.6 ± 1285.5	
	4	4364.7 ± 1716.6	

	8	515.2 ± 148.1	X^2 = 5.6451, *d.f.* = 4, *P* = 0.2273
	12	585.2 ± 132.1	
	16	3969.8 ± 1931.3	
Limonene	0	500624.7 ± 170581.1	
	4	1632746.9 ± 1022844.8	
	8	349326.1 ± 124792.3	X^2 = 20.0476, *d.f.* = 4, *P* = 0.0005
	12	287922.5 ± 131844.7	
	16	192089.3 ± 51886.1	
Myrcene	0	243409.2 ± 105504.2	
	4	357663.7 ± 143272.8	
	8	126246.2 ± 68605.9	X^2 = 18.7106, *d.f.* = 4, *P* = 0.0009
	12	163509.9 ± 115985	
	16	71354.2 ± 24777.1	
Pinocamphone	0	5307.5 ± 1257.4	
	4	4973.7 ± 1252.8	
	8	1898 ± 729.8	X^2 = 26.1253, *d.f.* = 4, *P*<0.0001
	12	2335.7 ± 851.2	
	16	2354.3 ± 567.8	
Pinocarvone	0	1966.4 ± 522.4	
	4	1875.8 ± 483.3	
	8	877.5 ± 255.9	X^2 = 27.7285, *d.f.* = 4, *P*<0.0001
	12	593.6 ± 177.3	
	16	707.2 ± 197.6	
Terpinolene	0	31876 ± 10225.7	
	4	49021.2 ± 21863.4	
	8	10750.3 ± 4549.7	X^2 = 27.1102, *d.f.* = 4, *P* < 0.0001
	12	13182.1 ± 6380.5	

	16	8536.4 ± 2517.3	
trans-Anethole	0	12515.9 ± 4939.4	
	4	18767.2 ± 10869.2	
	8	3205 ± 1151.3	X^2 = 31.5659, *d.f.* = 4, *P* < 0.0001
	12	3881.6 ± 1369.9	
	16	1948.9 ± 592.7	
trans-Pinocarveol	0	3787.8 ± 783.9	
	4	6733.7 ± 3271.1	
	8	1664.8 ± 434.3	X^2 = 25.1396, *d.f.* = 4, *P* < 0.0001
	12	1467.7 ± 363.8	
	16	2396.1 ± 662.7	
Tricyclene	0	19824.9 ± 5021.7	
	4	28242.3 ± 14247.8	
	8	7985.3 ± 2172.7	X^2 = 25.0606, *d.f.* = 4, *P* < 0.0001
	12	8407.1 ± 3452.1	
	16	8895.6 ± 2096.3	
Verbenene	0	16300.6 ± 2894.5	
	4	11232.9 ± 1869.3	
	8	5146.1 ± 1149.4	X^2 = 19.1291, *d.f.* = 4, *P* = 0.0007
	12	9753.9 ± 3622.2	
	16	9552.9 ± 3435	

FIGURE LEGEND

Fig. 3.1. Observation chamber used to monitor behavioral activity of *Ibalia leucospoides ensiger*.

Fig. 3.2. Ovipositor insertion rates of *Ibalia leucospoides ensiger* in response to water, *Sirex* venom, and *Sirex* oviposition holes over five time periods.

Fig. 3.3. Response of female *Ibalia leucospoides ensiger* antenna to SPME-collected volatiles from a two-week old *Sirex nigricornis* oviposition site.

Fig. 3.4. Response of *Ibalia leucospoides ensiger* antennae to a synthetic mixture of compounds identified in SPME-collected samples collected from a two week old *Sirex nigricornis* oviposition site.

Fig. 3.5. Dendrogram of SPME-collected volatiles from a two week old *Sirex nigricornis* oviposition site in A. 0 days and B. 16 days after the logs were inoculated. C = control, M = *Sirex* venom, L = *Sirex* oviposition site; 1-4 = replication.

Fig 3.1

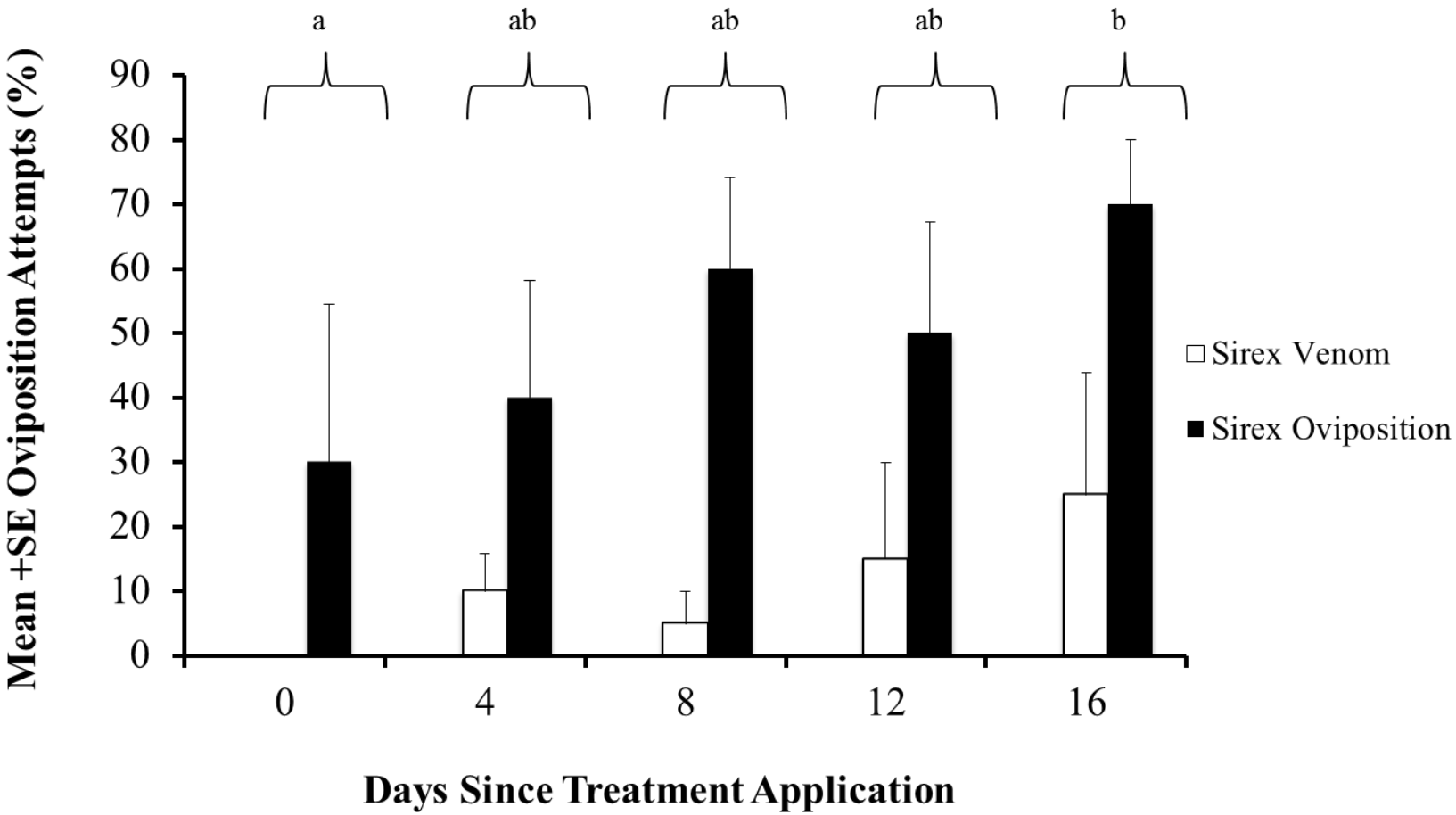

a
ab
ab
ab
b
90
80
70
60
50
40
30
20
10
0
Mean +SE Oviposition Attempts (%)
0
4
8
12
16
Days Since Treatment Application
Sirex Venom
Sirex Oviposition

Fig. 3.2

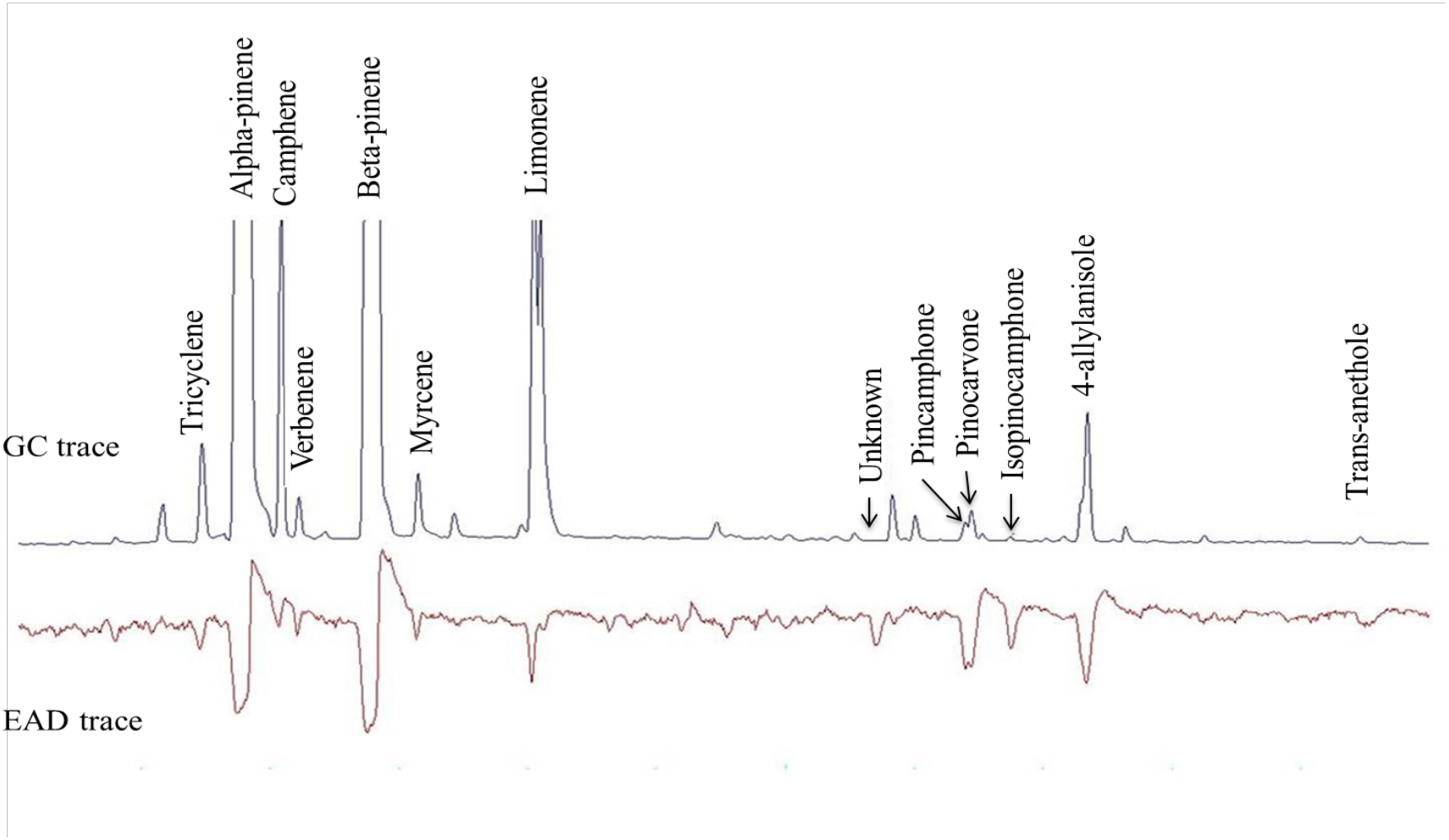

GC trace
EAD trace
Tricyclene
Alpha-pinene
Camphene
Verbenene
Beta-pinene
Myrcene
Limonene
Unknown
Pincamphone
Pinocarvone
Isopinocamphone
4-allylanisole
Trans-anethole

Fig. 3.3

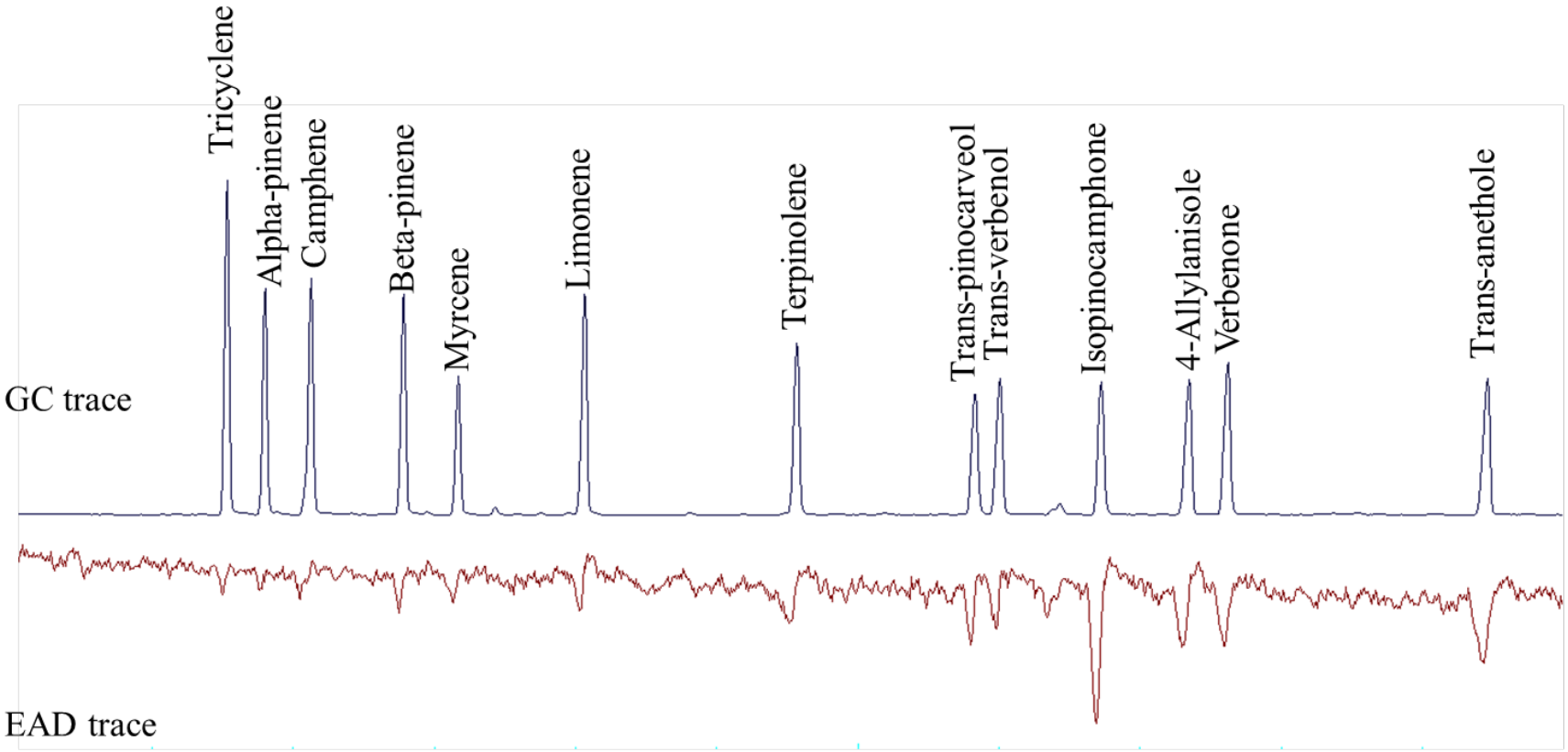
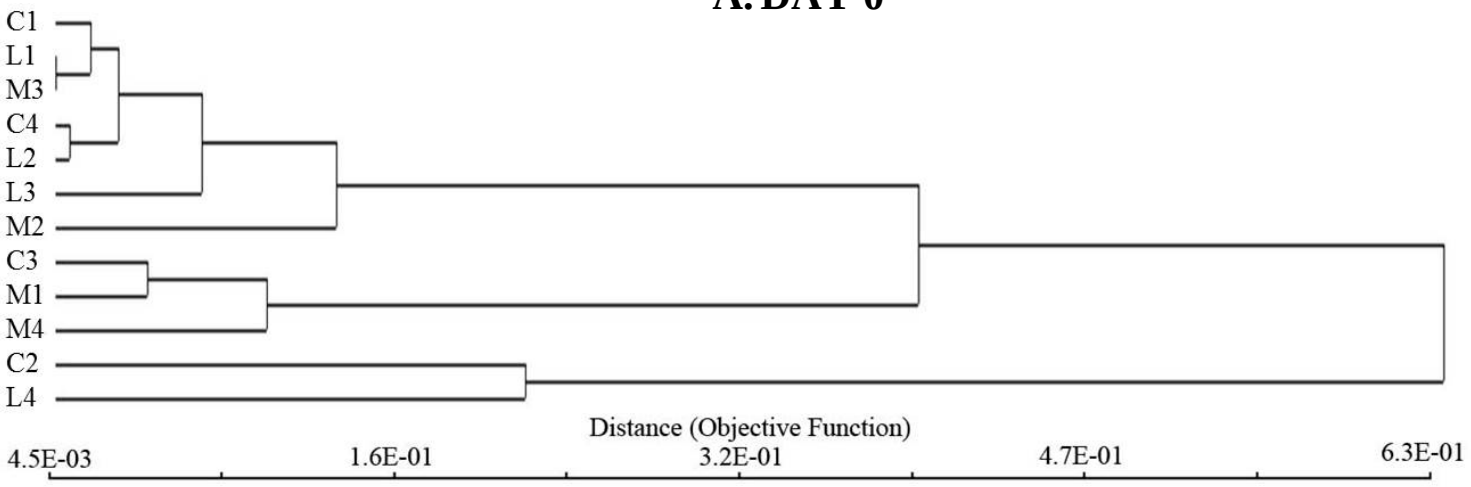

Tricyclene
Alpha-pinene
Camphene
Beta-pinene
Myrcene
Limonene
Terpinolene
Trans-pinocarveol
Trans-verbenol
Isopinocamphone
4-Allylanisole
Verbenone
Trans-anethole
GC trace
EAD trace

Fig. 3.4

A. DAY 0



B. DAY 16

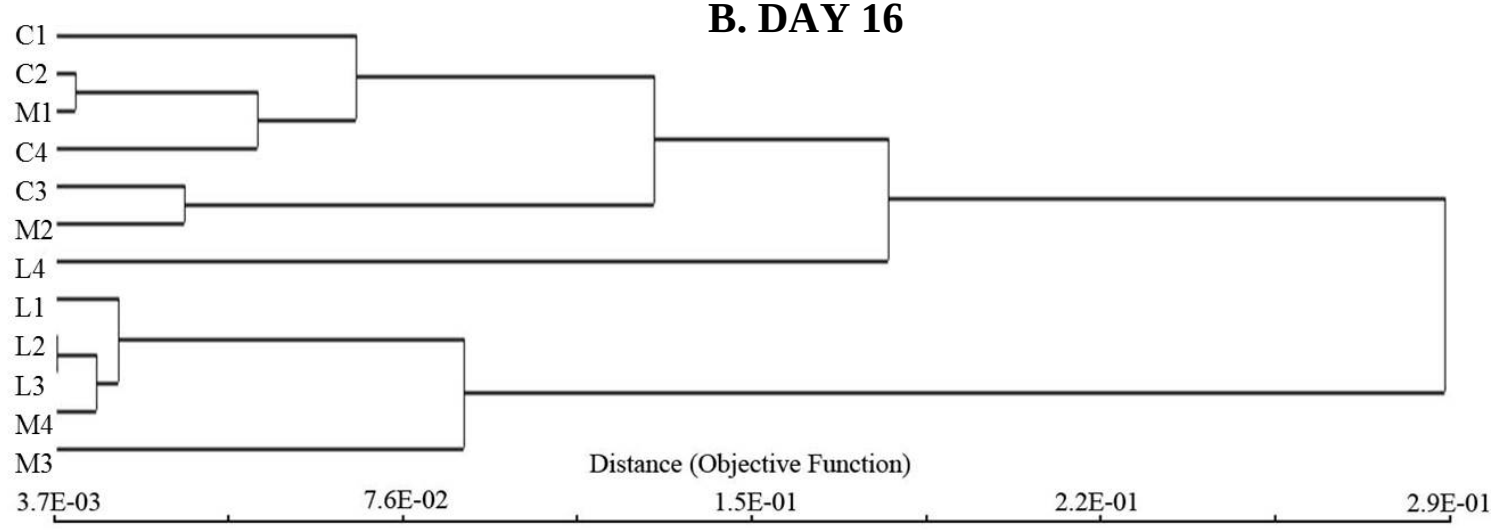


Fig. 3.5

CHAPTER 4

THESIS CONCLUSIONS

Sirex noctilio F. is an invasive woodwasp capable of destroying millions of dollars' worth of commercial timber. An effective response to manage this economic and ecological threat is vital to ensuring that this insect infestation remains contained, if not eradicated from landscapes. *Ibalia leucospoides ensiger* Norton is one of the few hymenopteran parasitoid species that has been shown to become established in an area of *S. noctilio* infestation. The ability to increase the numbers of parasitoids in response to an infestation can compound the effectiveness of the biocontrol agent.

Results from Chapter 2 indicate that there is a difference between the types and quantity of sensillum present on male and female *I. l. ensiger*. The differences in antennal composition suggest that each sex responds to different stimuli. The roles of the males are to mate with females and little, if anything, else. The females need to locate a suitable host and determine its viability. This in itself requires large amounts of data to be interpreted with specific sensors. Further research into the functionality of each type of sensillum on this insect may help with determining which type of sensory input is the most important to the acquisition of a targeted host.

Chapter 3 identified 14 compounds associated with highly behaviorally-active sources (*Sirex* oviposition sites) which elicited a response by *I. l. ensiger* through electroantennography (EAG). The behavioral assay showed that there was no interest

produced by an artificial drill hole in the tree and that other components were present in association with natural oviposition sites that elicited oviposition attempts. The venom-only treatment did elicit parasitism attempts but oviposition sites by *S. noctilio* produced the greatest response. Responses by female *I. l. ensiger* increased over time, which supports previous literature. With regard to the time of parasitism in response to oviposition by *S. noctilio*, our study diverges from previous literature. Our study showed that there are parasitism attempts immediately (same day) after *Sirex* oviposition. The symbiotic fungus of *Sirex* spp. which data suggests is producing host location cues for *I. l. ensiger* presumably had had little time to grow. We hypothesize that there is an interaction effect between the venom and fungal component, when combined, may be producing volatiles inducing the greatest response by parasitoids.

Future research using the results obtained from this study may begin with analyses of volatiles using GC-MS with combined fungus and venom. These results compared to pure venom and pure fungal inoculations may show if there is truly an interaction effect in place. Olfactormeter studies in the laboratory with specific compounds followed field testing may yield further details about the responses of *I. l. ensiger* to host location cues.