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Male-Produced Pheromone in the European Woodwasp, *Sirex noctilio*

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Abstract A male-produced pheromone that attracts both males and females was identified for the European woodwasp, *Sirex noctilio*, a serious pest of pine trees. Males displayed excitatory behaviors when placed in groups, and were attracted to the odors from males that were 2-5-d-old, but not to odors from males that were 0-1-d-old. An unsaturated short-chain alcohol, (*Z*)-3-decen-1-ol, was discovered in samples collected on SuperQ filters over groups of males and identified by using micro-derivatization reactions and gas chromatography coupled with mass spectrometry (GC-MS). The compound was not detected in volatile samples from females. Gas chromatography coupled electroantennographic detection (GC-EAD) of antennae from males exposed to male headspace odors produced strong antennal responses to the main peak of (Z)-3-decen-1-ol, as well as to an unknown minor component that had a similar retention time. Antennae from both males and females responded to synthetic (*Z*)-3-decen-1-ol. Several different synthetic candidates for the GC-EAD active minor components were selected based on GC-MS and GC-EAD responses to male headspace collections. These synthetic compounds were tested for antennal activity using GC-EAD, and those that produced strong responses were blended with the major component and tested for male attraction in the Y-tube olfactometer at different concentrations and ratios. Males tested in the Y-tube olfactometer were attracted to a synthetic blend of (*Z*)-3-decen-1-ol and (*Z*)-4-decen-1-ol at a ratio of 100:1. Whereas the addition of some suspected minor compounds reduced attraction, the addition of a third compound found in male emanations that produced strong male antennal responses, (*E,E*)-2,4-decadienal (at a ratio of 100:1:1), resulted in attraction of both males (Y-tube and wind tunnel) and females (wind tunnel).

Key Words Pheromone · Y-tube Olfactometer · Wind tunnel · Attractant · Wood wasp · Hymenoptera · Siricidae · Invasive insect

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Introduction

The European woodwasp, *Sirex noctilio* F. (Hymenoptera: Siricidae) has become a major pest of pines outside of its native range of Eurasia and northern Africa due to human-assisted transport of infested wood into those areas where susceptible host species grow. The wasp, introduced to New Zealand around 1905 (Coutts, 1965), spread to Tasmania, Australia, Brazil, Uruguay, Argentina, South Africa, and Chile (Smith and Schiff, 2002; Ciesla, 2003; Collett and Elms, 2009). Most recently, it was discovered in the northeastern United States and subsequently in Canada (Hoebeke et al., 2005; Collett and Elms, 2009). Its introduction into the United States is of concern because, in pine plantations

in other parts of the world, it is a serious pest of pine species native to North America (Carnegie et al., 2006).

The adult female has a long serrate ovipositor used to drill into the sapwood of the host tree (Madden, 1974). During oviposition, she also deposits a symbiotic fungus, *Amylostereum areolatum* (reviewed by Slippers et al., 2003) and a phytotoxic mucus, which together can kill the tree. Larvae feed and develop in the wood, and rely on the fungus to aid in the digestion of cellulose (Rawlings, 1953; Gilmour, 1965). Typically, larvae overwinter inside the trees and emerge the following summer (Zondag, 1969). Adult wasps live up to 12 d (Zondag, 1969), and have haplo-diploid sex determination (Rawlings, 1953) in which unmated females produce unfertilized eggs that develop into haploid males. Mated females can deposit either unfertilized eggs that become males, or fertilized eggs that develop into diploid females.

Until recently, most chemical ecology research on *S. noctilio* has been directed towards the discovery of host tree volatiles attractive to gravid females (Simpson, 1976; Simpson and McQuilkin, 1976). A female contact sex pheromone was discovered recently that elicits copulation attempts from males upon contact (Böröczky et al., 2009). However, little is known about how *S. noctilio* males and females find each other in the wild to mate. There have been reports of male *S. noctilio* swarming near the tops of trees (Madden, 1982; 1988). Such behavior suggests the presence of a male aggregation pheromone. In this study, we report production by male *S. noctilio* of a major compound, *(Z)*-3-decen-1-ol, and when blended with two minor components, *(Z)*-4-decenol and (*E,E*)-2,4-decadienal, the blend is attractive to both males and females in laboratory bioassays.

Methods and Materials

Staggering Adults for Emergence In upstate New York, *S. noctilio* adults start to emerge in the field around the beginning of July, and emerge over a period of several weeks (Zylstra et al., 2010). Adults live only 6–12 d (Madden, 1982). At the USDA APHIS PPQ facility in Syracuse, NY, USA (hereafter referred to as 'Syracuse laboratory'), infested pine logs are routinely taken in from the field at the end of the fall and kept in plastic barrels (0.6 m tall× 0.45 m diam.) with screen covers until adult emergence. Artificially increasing the number of degree days in the laboratory results in earlier emergence. In order to increase the window of time when adult wasps were available for behavioral bioassays at both the Syracuse laboratory and the USDA APHIS PPQ Otis Quarantine Laboratory, in Buzzards Bay, MA, USA (hereafter referred to as 'Otis laboratory'), the overwintering period was manipulated by bringing infested wood into the laboratory at different times during the winter. In the fall in Oswego county (2008, 2009) and Oneida county (2010), NY, Scots pine trees (*Pinus sylvestris* L.) that had been girdled prior to *S. noctilio* flight the preceding June, were felled, cut into bolts of about 0.6 m in length, and sealed at the ends (Waxlor End Sealant, Willamette Valley Co., Eugene, OR, USA).

For volatile collection studies conducted at the Syracuse laboratory in 2009, live insects were provided in two batches. Barrels of logs that were placed indoors at the end of 2008 and stored at approximately 25ºC produced adult males and females from February to March 2009. Barrels of logs that were brought inside in April 2009 produced adults from May to June 2009.

For the behavioral experiments performed in the quarantine facility at the Otis laboratory, a group of barrels was brought indoors on a monthly basis from November to March, from 2008 to 2011. The average environmental conditions for barrel storage were approximately 23.5ºC and 16% R.H. The barrels were monitored daily for *S. noctilio* emergence. By staggering when barrels full of logs were brought in from the cold over a period of 5 mo, a small but steady supply of *S. noctilio* adults were available for study between the end of January and mid-June.

Insects for Electrophysiology and Behavior As wasps emerged at the Otis Laboratory, they were placed individually in labeled, 18 ml plastic vials (Bioquip, Rancho Dominguez, CA, USA) with a piece of filter paper and a single air hole in the snap cap. Insects were not given an opportunity to mate. Vials of wasps were held in a walk-in environmental chamber at 23–24ºC and 42–61% R.H., with a light regime of 16:8 L:D, which was also used for behavioral bioassays. Vials of males and females were kept in separate plastic shoeboxes. Adults of *S. noctilio* are not known to feed, so no food was provided (Morgan and Stewart, 1966; Zondag, 1969). Wasps tested in behavioral bioassays were 0-5-d-old; wasps used in volatile collections were 0-10-d-old; and wasps used in electrophysiology were 2–6 d old. Bioassays were performed in the same walk-in environmental chamber between 0830 and 1600 h.

Lighting Bright lighting was used for odor collections and bioassays. At the Syracuse laboratory, a 250 W light source was used in addition to the ceiling lights. At the Otis laboratory, bright light was provided by 4 40 W Gro-Lux wide-spectrum fluorescent bulbs (Sylvania, Danvers, MA, USA) for odor collections and Y-tube olfactometer experiments, and 2 full spectrum fluorescent bulbs illuminated the working section of the wind tunnel (Aqualine T5 Reef White, 10 K, 54 W, 115 cm×16 mm, Aqua Medic, Germany). At the Otis Laboratory, light intensity measured above the aeration chambers and the Y-tube olfactometer was about 2,600 lux, and light intensity measured inside the

wind tunnel was about 6,600 lux at the ceiling, 5,050 lux at mid-height, and about 2,390 lux on the wind tunnel floor.

Volatile Collections In 2009 at the Syracuse laboratory, the first volatile collections were performed. Different numbers of live *S. noctilio* males or females (3–30) were kept in a Plexiglas® cage (61×46×46 cm), and volatile compounds were collected on SuperQ filters (30 mg/filter) by flushing the cage with charcoal-filtered air. Volatile collections started with the insect-free cage in the morning, then 3 males (or females) were added, after which the number of males (or females) was increased gradually to 10, to 20, and finally to 25–30. Plastic screening (70×50 cm) and Scots pine branches inside the cage afforded walking surfaces for the insects. Volatiles were collected for 30 min after which the filter was changed. The ends of used filters were sealed with Teflon tape, and the filters were wrapped in aluminum foil. Filters were eluted at the Penn State laboratory with 100 μl of a hexane:dichloromethane 1:1 mix.

Volatile collection experiments continued at the Otis laboratory in 2010 and 2011, and provided samples for both electrophysiological and chromatographic experiments. Virgin males or females, individually or up to 20, were placed in 4-L glass chambers with filter paper for collection of headspace volatiles. The headspace volatiles were sent through Teflon tubing to the Y-tube bioassay apparatus (described below), or collected using either a 100-μm polydimethylsiloxane-coated solid phase micro-extraction (SPME) fiber (Supelco, Bellefonte, PA, USA), a 20 mg activated carbon trap, or a 20 mg Porapak-Q trap (Analytical Research Systems, Inc., Gainesville, FL, USA). SPME fibers were conditioned between uses by baking for 45 min at the injection port temperature of 250°C. Charcoal and Porapak-Q traps were rinsed with 0.2 ml of either hexane, acetone, dichloromethane, or ethyl acetate. Solvents of differing polarity were used in attempts to separate unknown active volatile components. Volatile collection times ranged from 1 to 3,480 min. Control headspace volatiles were sampled from a chamber containing only filter paper. SPME fibers also were exposed to the headspace of individual males or females in 5 dram plastic vials for 1–95 min.

Y-tube Olfactometer Choice Bioassays A glass Y-tube olfactometer was used for choice bioassays inside the walk-in environmental chamber in the quarantine at the Otis laboratory. The olfactometer consisted of the glass "Y" (34 mm I. D.×20 cm base and 12 cm arms) with the two upwind arms at a 90º angle from each other (Analytical Research Systems, Inc., Gainesville, FL, USA). A Y-shaped piece of fiberglass window screen was cut 1 cm wide to fit inside the length of the Y-tube to allow wasps to have traction inside the glass tube. This was replaced with a clean piece between treatments. Air pressure was supplied by an oil-less air compressor. Air passed through a carbon filter, a splitter, bubbled through distilled water, through two flow meters set to 0.2 L/min, then through Teflon tubing attached to ports directly on the Y-tube olfactometer. In aeration bioassays, air traveled into the two 4-L glass aeration chambers prior to entering the Y-tube olfactometer. Cardboard was placed between the aeration chambers and the Y-tube, and white sheets were suspended around the sides of the Y-tube to remove visual directional cues.

Adult *S. noctilio* males were individually placed in the downwind entry port of the Y-tube olfactometer and were observed for 3 min. If the wasp travelled to the upwind 5 cm of one of the arms on the Y-tube olfactometer, its choice was recorded and the wasp was removed. If no choice was made within 3 min, it was marked as non-responsive and removed. In preliminary tests, the number of times the following behaviors commenced was recorded: resting, walking, grooming, vibrating abdomen, antennating, splaying of genitalia, flying, and fanning wings.

Preliminary tests revealed that males performed well in the Y-tube olfactometer, whereas females had difficulty maneuvering and rarely responded to odors in the confined tube. Subsequently, only males were used in the Y-tube choice tests. The first set of Y-tube bioassays involved odors naturally emitted by groups of males in two 4-L glass chambers. Air was pushed through those chambers and into the upwind arms of the Y-tube olfactometer. In the second set of bioassays, synthetic compounds in varying concentrations and combinations were applied to grey rubber septa (West Pharmaceutical Services, Kearney, NE, USA). The olfactometer was flipped every 3 replicates to avoid directional bias due to lighting or visual cues, and all glassware was cleaned between tests with Citranox odorless detergent (Alconox, Jersey City, NJ, USA) and hot water, rinsed with acetone, and baked at 140ºC for at least 1 h.

Gas Chromatography and Electrophysiology Antennal responses to odors were analyzed at the Otis laboratory using gas chromatography (GC) coupled with electroantennographic detection (EAD). An Agilent 6890 GC in splitless mode, initially with an HP-5 column (30 m×0.320 mm I. D.×0.25 μm film; Agilent Technologies, Inc., Santa Clara, CA, USA) and helium carrier gas, was used with a starting temperature of 50ºC for 0.75 min, ramped 20ºC/min to 250ºC, and then held for 15 min. In attempts to improve peak separation, this temperature program was made slower by changing the start temperature to 40ºC and the ramp to 10ºC/min, 5ºC per min, or 2.5ºC per min. The GC injector temperature was 250ºC, and the FID temperature was 275ºC. At the Otis Laboratory, both HP-5 (Agilent) columns and polar Stabilwax (Restek) columns with the same dimensions were used on both the GC-MS and GC-EAD in attempts to improve peak separation. Near the end of the

GC column, a glass Y-connector split the effluent to the flame ionization detector (FID) and to the EAD. The EAD effluent traveled through a temperature controlled arm (Syntech Temperature Controller, Kirchzarten, Germany) at 200ºC, and into the side of a glass L-shaped stimulus delivery tube (7 mm i.d.), then to the antennae. Charcoal-filtered air passed through a humidifier, a flow meter, and into the stimulus delivery tube with flow of 0.5 L/min.

Antennae were removed at the basal flagellomere, and the tip of the terminal flagellomere was cut off using a razor blade against a piece of filter paper moistened with insect saline (see Cooperband et al., 2008). Four antennae were inserted between glass capillary saline electrodes, placed at the end of the stimulus delivery tube, beneath an aluminum foil cover that acted as a faraday cage. Electrodes were connected to 2 mm-diam. capillary electrode holders (World Precision Instruments, Sarasota, FL, USA) that were held in place by magnetic micropositioners (Signatone Corp., Gilroy, CA, USA) on a stainless steel platform (Syntech, Kirchzarten, Germany). The whole platform was placed on a lab jack that allowed for vertical positioning of the antennal preparation at the end of the stimulus delivery tube. All metal objects were grounded.

The signal from the antennae was first amplified and filtered (Grass Amplifier Model P55, Astro-Med, Inc., West Warwick, RI, USA) and then filtered again (HumBug, Quest Scientific, North Vancouver, BC, Canada). That signal and the GC signal were sent to a six-channel integrator (Model 302, SRI International, Menlo Park, CA, USA). PeakSimple v. 3.85 software (SRI International, Menlo Park, CA, USA) was used to capture the data.

Chemical Identification and Synthesis of the Main Component An Agilent 6890 N GC coupled with a 5973 mass-selective detector (GC-MS) equipped with an Equity-5 capillary column (30 m×0.2 mm×0.2 μm; Supelco, Bellefonte, PA, USA) was used to analyze odor samples at the Penn State laboratory. The oven program started at 45°C, held for 1 min, followed by a temperature ramp of 10°C/min to 300°C, held for 5 min. The temperature of the injector and the transfer line was held at 260°C and 300°C, respectively. Helium was used as a carrier at an average linear velocity of 30 cm/s, and samples were injected splitless after which the split valve was opened at 0.75 min. The MS was operated in the electron impact (EI) mode. Compounds were identified based on their mass spectra (NIST 05) and Kovats indices (Van Den Dool and Kratz, 1963; Kovats, 1965).

Micro-scale epoxidation reaction (Attygale 1998) was performed on the major male specific compound, decen-1-ol, that was detected in the Super Q aeration samples to determine the double bond position and geometry. To 100 μl of an aeration sample, 100 μl of a 0.2 mg/ml solution of *m*-chloroperbenzoic acid (*m*-CPBA) in dichloromethane were added, and the reaction mixture was held at room temperature for an hour in a closed 1-ml screw-cap vial. The mixture then was washed with 10% solution of sodium carbonate and the phases were separated. The solvent was evaporated under a gentle stream of nitrogen from the organic phase, and the residue was resuspended in 50 μl of hexane. The reaction was performed on two aeration samples and the position of the double bond was determined by examining the EI-MS fragmentation pattern of the epoxide.

To synthesize (*Z*)-3-decen-1-ol a sample of commercially available 3-decyn-1-ol (ALFA Aesar, Ward Hill, MA, USA) was hydrogenated over P-2 nickel (Brown and Ahuja, 1973) at atmospheric pressure (conducted at Virginia Military Institute by THJ). Analysis by GC-MS showed this product to be >99% pure with a small amount (<1%) of decanol present.

Identification of Minor Components Minor component peaks were difficult to distinguish due to their presence in minute quantities and their similarity in retention time to the main component. However, male antennal responses in GC-EAD revealed a second strong antennal response immediately after the response to the main component. Based on the GC-MS results using the NIST database at the Otis Laboratory, a list of 12 tentative compounds was compiled, and those compounds were acquired commercially (Sigma-Aldrich) or synthesized (THJ). Synthetic suspected compounds were serially diluted to 100 ng/μl in hexane, and tested for male antennal activity using 0.2 to 1.0 μl injections in the GC-EAD. After testing tentative minor compounds, the major component was tested on the same antennae to verify the antennal preparation was still functioning and responsive. Tentative minor compounds that produced strong male antennal responses were tested for male attraction at varying concentrations and blends in the Y-tube olfactometer against hexane controls, using gray rubber septa (West Pharmaceutical Services, Kearney, NE, USA).

Wind Tunnel Choice Bioassays The two blends that attracted males in the Y-tube olfactometer were tested in a wind tunnel to evaluate attraction in both males and females. A laminar flow, push-pull wind tunnel, with a working section of 120 cm long×91 cm tall×75 cm deep, was used, with an air speed of 35–45 cm/s. The wind tunnel was located inside a walk-in environmental chamber and operated at 23.5°C and 50% R.H. Air from the environmental chamber passed through an activated carbon honeycomb filter (50.8×63.5×5 cm, 4.9 kg activated carbon per m^2, Air Handler, Niles, IL, USA) before entering the wind tunnel. Air exiting the tunnel passed through an additional activated carbon filter for cleaning before returning to the environmental chamber (50.8×63.5×2.5 cm, 3.4 kg activated carbon per m^2, Air Handler, Niles, IL, USA).

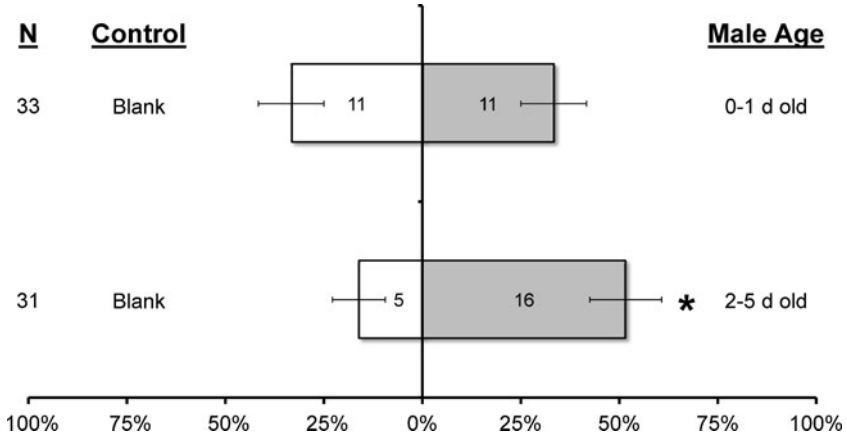

Fig. 1 Individual male *Sirex noctilio* tested in the Y-tube olfactometer were given a choice of odors emanating from either a blank jar or a jar with groups of males of different ages. *Asterisk* indicates significant difference between choices at α=0.05; *error bars* represent standard errors; *numbers in bars represent* number of individuals making each choice

Taking advantage of the phototactic nature of *S. noctilio* (Madden, 1974), two rubber septa lures in wind tunnel bioassays were hung 3.5 cm below the ceiling of the wind tunnel at the upwind end of the tunnel, and approximately 15 cm apart. A 5 cm disc cut from a black panel trap (AlphaScents, Bridgeport, NY, USA) was suspended directly below each lure. Male or female *S. noctilio* adults were released individually at the downwind end of the wind tunnel and observed for 3 min per trial. Each wasp was observed for upwind flight, whether they landed on or touched one of the two targets, and which target was chosen.

Statistical Analysis Dual choice bioassays conducted in the Y-tube olfactometer and in the wind tunnel were used to test the null hypothesis that both stimuli were chosen at the same frequency. Results were analyzed using the *Chi Square* Goodness-of-fit test. Significant differences between sides occurred when the test statistic $G \geq 3.841$ (df=1, α=0.05) (Sokal and Rohlf, 1995).

Results

Staggered Emergence Trees used in 2009 were naturally infested, whereas trees used in 2010 and 2011 were healthy trees that had been girdled prior to *S. noctilio* flight. Infested logs brought into the Otis Laboratory in five groups staggered over 5 months yielded 124, 388, and 735 *S. noctilio* wasps, in 2009, 2010, and 2011, respectively. These supplied the wasps used in aerations, electroantennograms, and behavioral bioassays at the Otis Laboratory. The sex ratios (males/total) for the respective years were 0.82, 0.73, and 0.70.

Male Response to Males When males were placed in groups of three or more in the 4-L aeration chamber, they were observed to engage in increased walking and flying while simultaneously everting their genitalia. These behaviors were also observed when placed in the Y-tube olfactometer. Additional behaviors observed during Y-tube experiments included vibrating their abdomen or antennae, and fanning their wings. Males were attracted to odors from groups of males that were 2-d-old or more, but not groups of males that were 1-d-old or less (Fig. 1).

Identification of Main Pheromone Component Headspace samples analyzed using GC-MS and GC-EAD revealed that groups of males 2-d-old or more released major amounts of a compound not detected in female emanations, or from males less than 2-d-old (Table 1). Of 12 individual males in vials that were sampled by SPME, two individuals produced the major component.

The main male specific compound found in the SuperQ samples has a Kovats index of 1,256 (on the Equity-5 column) and the EI-MS spectrum matched well (90%) with a few decen-1-ol isomers. The fragment *m/z* 138 indicated loss of water. The very low intensity of *m/z* 128 and the relatively low intensity of *m/z* 57 compared to that of *m/z* 55 suggested it was not decanal, which otherwise has the same molecular mass (156). There were no alkyl fragments or loss of alkyl fragments indicating branching of the chain. In order to determine the position of the double-bond, two samples containing the alcohol were subjected to epoxidation. Mass spectral analysis of the derivative showed

Table 1 Number of aerations at the Otis laboratory that contained (*Z*)-3-decen-1-ol out of total number of aerations conducted with males and females of different ages, either in groups or individually

Age	Grouping	No. of aerations	
		Male	Female
<2 d old			
	Single	0/10	0/1
	Multiple	–	0/2
2+ d old			
	Single	2/7	0/2
	Multiple	21/26	0/9

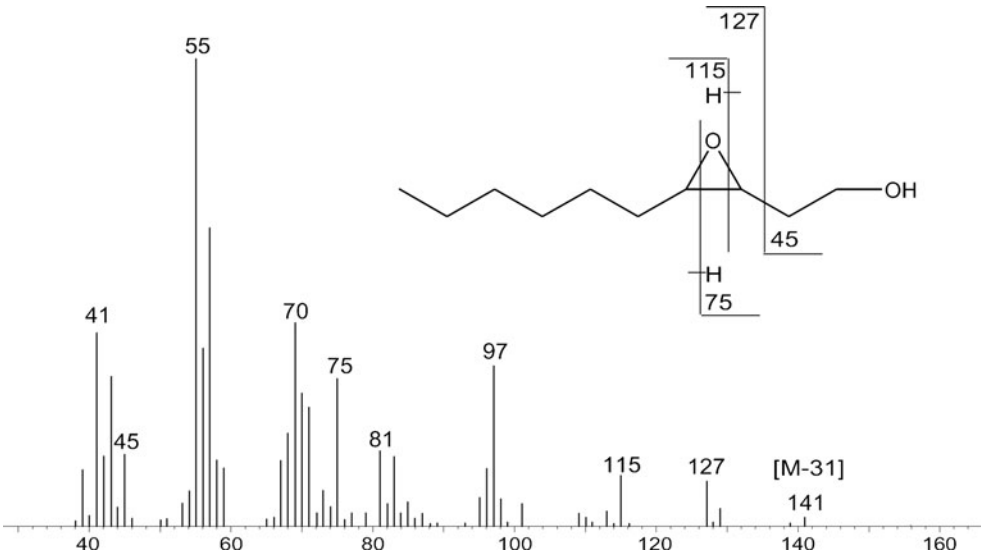

Fig. 2 EI-MS spectrum of the epoxide of (*Z*)-3-Decen-1-ol

diagnostic ions at *m/z* 127 and 45 corresponding to fragments formed by α-cleavage (adjacent to the epoxide group) on the alcohol-end of the molecule (Fig. 2). Transannular fragmentation resulted in the characteristic ions $C_7H_{15}CH{=}OH^+$ and $HOC_2H_4CH{=}OH^+$ at *m/z* 115 and 75, respectively. Thus, the double bond was identified to be at the 3rd position. The (*Z*)-isomer of 3-decen-1-ol was synthesized, as described above, and was used to determine the geometry of the double bond of the natural product. The retention times of the epoxide derivative of the synthetic (*Z*)-3-decen-1-ol and that of the natural product on the Equity-5 column matched perfectly, thus confirming the (*Z*)-geometry. No (*E*)-isomer of the synthetic standard was observed after derivatization.

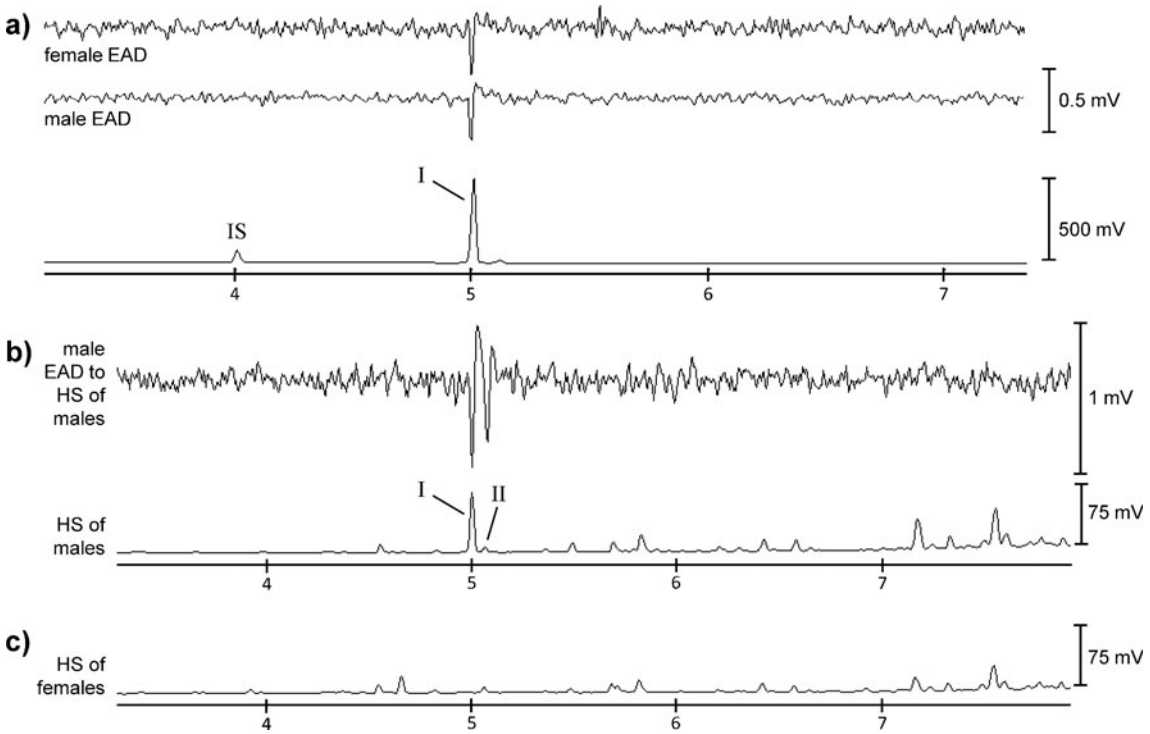

Fig. 3 Chromatographic traces of **a** GC-EAD showing no response to 5 ng of the internal standard (*E*)-cyclodecene (IS), and positive antennal responses by both male and female *Sirex noctilio* to 50 ng of synthetic (*Z*)-3-decen-1-ol (I); **b** GC-EAD of odors collected for 870 min on SPME fiber from the headspace of 4 *S. noctilio* males showing two typical male antennal responses to HS components (I) (*Z*)-3-decen-1-ol, and (II) unknown minor component; and **c** GC trace of odors collected for 870 min on SPME fiber from the headspace of 4 *S. noctilio* females, for comparison. *Tick marks* represent minutes of elution time on an HP-5 column with temperature program starting at 50°C and ramping 20°C/min to 250°C

Antennal Responses to Natural and Synthetic Compounds Strong antennal responses were elicited from both male and female antennae exposed to 10 to 100 ng of synthetic (*Z*)-3-decen-1-ol in the GC-EAD at the Otis Laboratory (Fig. 3a). When natural male headspace odor was tested against male antennae in the GC-EAD, two strong reproducible antennal responses were elicited: one response to (*Z*)-3-decen-1-ol, and another response immediately after the first, apparently in response to an unknown minor component that occurred in much smaller amounts (Fig. 3b). In comparison, the large peak of (*Z*)-3-decen-1-ol was absent from headspace odors collected from females (Fig. 3c).

Several smaller antennal responses corresponded to other small peaks from male odors. Using Kovats indices and peak shapes, male headspace peaks that elicited antennal responses in the GC-EAD were tentatively matched to male headspace peaks in the GC-MS, which was used to compile a list of possible identities for unknown minor compounds. The synthetic versions of those tentative compounds were obtained, and retention times of synthetic compounds were compared to the male headspace GC-MS results. The list of suspected minor components is given in Table 2, along with retention times and a summary of antennal responses.

The average ratio of the main active component and the peak suspected of being the minor component in SPME samples was 1000:8 (N=11). The minor component produced a very strong male antennal response despite its minute quantity.

Bioassays with Synthetic Blends The synthetic (*Z*)-3-decen-1-ol was serially diluted in hexane and applied in aliquots of 100 μl to rubber septa. Five concentrations were tested for

Table 2 Retention times (RT) of synthetic compounds using the HP-5 column and the polar Stabilwax column, compared to RT of odors of interest from headspace (HS) collections of male *Sirex noctilio*. For comparison, the major component is in *bold* and *shaded gray*. Kovats indices for synthetic compounds are also provided, as well as intensity of male antennal responses to synthetic compounds (strong, weak/inconsistent, or not detected)

	Kovats Index of Synthetic Compound		RT (min) of Synthetic Compound		Male EAD Response to Synthetic Compound[c]	RT (min) of Unknown Peak of Interest from HS		Synthetic RT Matches HS RT[d]
	Column		Column			Column		
Compound[a]	HP-5	Stabilwax[b]	HP-5	Stabilwax[b]		HP-5	Stabilwax[b]	
α-Pinene	939		2.64		*	2.63		Y
β-Pinene	980		2.95		*	2.96		Y
(+)-α-Longipinene		1376		4.97	n.d.		4.97	Y
Nonanal		1399		5.08	***		5.08	Y
Verbenol		1556		6.06	*		5.93	N
(1S)-(-)-Verbenone		1633		7.06	*		7.06	Y
Myrtenol		1686		6.83	n.d.		7.30	N
(*E*)-3-Decen-1-ol	1250		4.95		***	5.06[e]		N
(*Z*)-5-Decen-1-ol	1256		4.98		***	5.06[e]		N
(*Z*)-3-Decen-1-ol	**1255**	**1671**	**4.99**	**6.74**	***	**4.95-5.08[f]**	**6.71-6.83[f]**	**Y**
(*Z*)-4-Decen-1-ol	1259	1680	5.01	6.79	***	5.06[e]	6.80[e]	Y
9-Decen-1-ol	1266		5.06		n.d.	5.06[e]		Y
(*E,E*)-2,4-Decadienal[b]	1319	1823[b]	5.42	6.89[b]	***	5.43	6.90[b]	Y

[a] Compounds were matched between the GC-EAD and GC-MS equipped with a DB-5 or Stabilwax column using Kovats indices and shape, identified by GC-MS, and purchased from Sigma-Aldrich (St. Louis, MO, USA), except for (*E*)-3-Decen-1-ol, (*Z*)-5-Decen-1-ol, and (*Z*)-3-Decen-1-ol which were synthesized by THJ. All data in this table used a temperature program starting at 50°C for 0.75 min, ramping at 20°C/min to 250°C. Slower temperature programs used in attempts to improve peak separation are not shown

[b] Data shown for (*E,E*)-2,4-Decadienal on the Stabilwax column were collected on the instrument used for GC-MS. Data shown for the other compounds were collected on the instrument used for GC-EAD

[c] Three asterisks indicate a consistently strong antennal response, one asterisk indicates a weak or inconsistent response, n.d. indicates that an antennal response was not detected

[d] RT matches within 0.03 min

[e] Unknown peak of interest was hidden under large natural peak of (*Z*)-3-Decen-1-ol, but RT from antennal response was used as an estimate

[f] The peak of (*Z*)-3-Decen-1-ol was approximately 0.12 min wide in samples containing enough material to identify smaller peaks, thus concealing any smaller peaks that eluted during that time

male attraction in the Y-tube olfactometer. Responses by males to dilutions of (*Z*)-3-decen-1-ol alone did not differ significantly from the control, however, the least negative response was to 100 μg (*Z*)-3-decen-1-ol, so this concentration was used in other tests (Fig. 4a). All Y-tube tests conducted with synthetic compounds, and their statistical analyses, are summarized in Table 3. Only three blends tested were significantly different from the control.

When offered two-component blends of (*Z*)-3-decen-1-ol in combination with 1 μg of (*E*)-3-decen-1-ol, (*Z*)-4-decen-1-ol, (*Z*)-5-decen-1-ol, (*E,E*)-2,4-decadienal, or nonanal, males were attracted to the two-component blend of 100:1 μg of (*Z*)-3-decen-1-ol and (*Z*)-4-decen-1-ol, but to none of the other blends. When offered a two-component blend of 10:1 μg of (*Z*)-3-decen-1-ol and nonanal, males chose the hexane control septum significantly more than the blend (Fig. 4b). When (*Z*)-3-decen-1-ol was tested for male attraction with different concentrations of (*Z*)-4-decen-1-ol, the only ratio tested that was chosen significantly more than the hexane control was 100:1 μg (*Z*)-3-decen-1-ol: (*Z*)-4-decen-1-ol (Fig. 4c).

When 1 μg of a minor compound was added to the attractive 2-component blend of 100:1 μg (*Z*)-3-decen-1-ol and (*Z*)-4-decen-1-ol, the resulting three-component blend of 100:1:1 μg of (*Z*)-3-decen-1-ol, (*Z*)-4-decen-1-ol, and (*E, E*)-2,4-decadienal produced significant attraction by males. However, the addition of 1 μg of (*E*)-3-decen-1-ol or nonanal to the attractive 2-component blend removed the attraction. The addition of 1 μg of (*Z*)-5-decen-1-ol or nonanal to the attractive 3-component blend also removed attraction (Fig. 4d).

Wind tunnel flights and choices when males and females were offered either the 2- or 3-component attractive blends are shown in Fig. 5. Both males and females had increased upwind flight, increased choices (by landing on or touching either target), and increased correct choices (blend vs. hexane control) when the three-component blend was presented rather than the two-component blend.

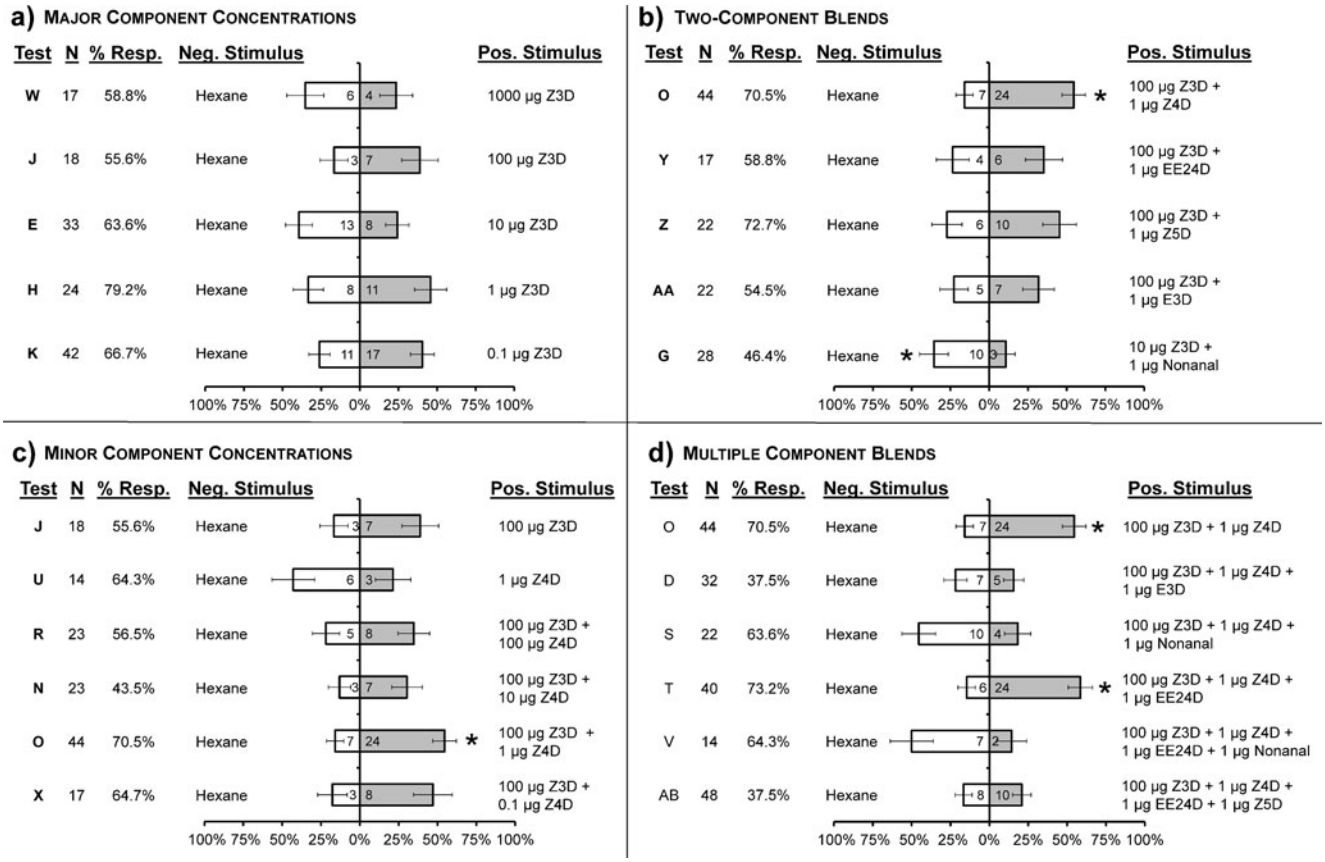

Fig. 4 Behavioral responses by male *Sirex noctilio* in the Y-tube olfactometer when presented with **a** different concentrations of (*Z*)-3-decen-1-ol (Z3D); **b** 2-component blends of (*Z*)-3-decen-1-ol in combination with 1 μg of either (*E*)-3-decen-1-ol (E3D), (*Z*)-4-decen-1-ol (Z4D), (*Z*)-5-decen-1-ol (Z5D), (*E,E*)-2,4-decadienal (EE24D), or nonanal; **c** (*Z*)-3-decen-1-ol and different concentrations of (*Z*)-4-decen-1-ol; **d** the attractive blends of 100 μg of (*Z*)-3-decen-1-ol and 1 μg of (*Z*)-4-decen-1-ol when combined with 1 μg of either (*E*)-3-decen-1-ol, nonanal, or (*E,E*)-2,4-decadienal. Percent responding (% Resp.) refers to the frequency that a choice was made. *Asterisk* indicates significant difference at α=0.05; *error bars* represent standard errors; *numbers in bars* represent number of individuals making each choice

Table 3 Tests conducted in the Y-tube olfactometer to evaluate attraction of male *Sirex noctilio* in response to different synthetic blends. *Percent* response refers to how many males travelled upwind in the Y-tube and made a choice. If the direction of the choices made were statistically significant, the direction towards the hexane control (Neg.) or the test blend (Pos.) is indicated. Compound amounts in blends tested are shown in μg per gray rubber septum

Test	Amounts of compounds (μg) in blend								*N*	% Resp.	*Chi-Sq.*	Choice
	Z3D	E3D	Z5D	Z4D	EE24D	Nonanal	α-pinene	β-pinene				
A	100	10		10					18	44.4%	0.0000	
B	10	1		1					28	50.0%	2.6566	
D	100	1		1					32	37.5%	0.3349	
E	10								33	63.6%	1.2020	
F	10					1	1	1	17	58.8%	1.6457	
G	10					1			28	46.4%	3.9765	Neg.
H	1								24	79.2%	0.4757	
I							1	1	32	62.5%	0.2003	
J	100								18	55.6%	1.6457	
K	0.1								42	66.7%	1.2957	
L	100							3	26	53.8%	0.2867	
M	100		10						20	50.0%	0.4027	
N	100			10					23	43.5%	1.6457	
O	100			1					44	70.5%	9.8572	Pos.
P	100			30					19	47.4%	0.1113	
R	100			100					23	56.5%	0.6986	
S	100			1		1			22	63.6%	2.6566	
T	100			1	1				41	73.2%	11.5647	Pos.
U				1					14	64.3%	1.0194	
V	100			1	1	1			14	64.3%	2.9419	
W	1,000								17	58.8%	0.4027	
X	100			0.1					17	64.7%	2.3583	
Y	100				1				17	58.8%	0.4027	
Z	100		1						22	72.7%	1.0107	
AA	100	1							22	54.5%	0.3349	
AB	100		1	1	1				48	37.5%	0.2227	

Z3D=(*Z*)-3-Decen-1-ol, E3D=(*E*)-3-Decen-1-ol, Z5D=(*Z*)-5-Decen-1-ol, Z4D=(*Z*)-4-Decen-1-ol, EE24D=(*E,E*)-2,4-Decadienal

Discussion

Since the first discovery of pheromones in the silk moth, *Bombyx mori* (Lepidoptera: Bombycidae) (Butenandt et al., 1959), most pheromone identifications have been in the Lepidoptera, Coleoptera, and social insects (Robacker and Hendry, 1977). In addition to the social Hymenoptera, volatile sex pheromones have been discovered in several families of the Apocrita: Eulophidae (Consoli et al., 2002), Eurytomidae (Leal et al., 1997), Ichneumonidae (Vinson, 1972), Pteromalidae (Yoshida, 1978), and Trichogrammatidae (Pompanon et al., 1997). In the Symphyta, however, few examples of volatile pheromones are available: Cephidae (Cossé et al., 2002), and Diprionidae (Jewett et al., 1978).

The pheromone described in this study likely functions as a lekking pheromone in *S. noctilio*. Such a pheromone would facilitate the formation of male swarms for females to fly through to mate. Similar behaviors have been described in other Hymenoptera (Marshall and Alcock, 1981). Although EAD of male headspace only reliably revealed two large antennal depolarizations, all three compounds (i.e., (*Z*)-3-decen-1-ol, (*Z*)-4-decen-1-ol, and (*E,E*)-2,4-decadienal) are important and act together synergistically. Strong antennal responses were elicited from all three synthetic compounds. Some of the other compounds found in the male headspace could have originated from the host tree, since many of them are known plant odors. Although nonanal produced strong antennal responses, adding it to the attractive blend reversed attraction, and it appeared to have a repellent effect.

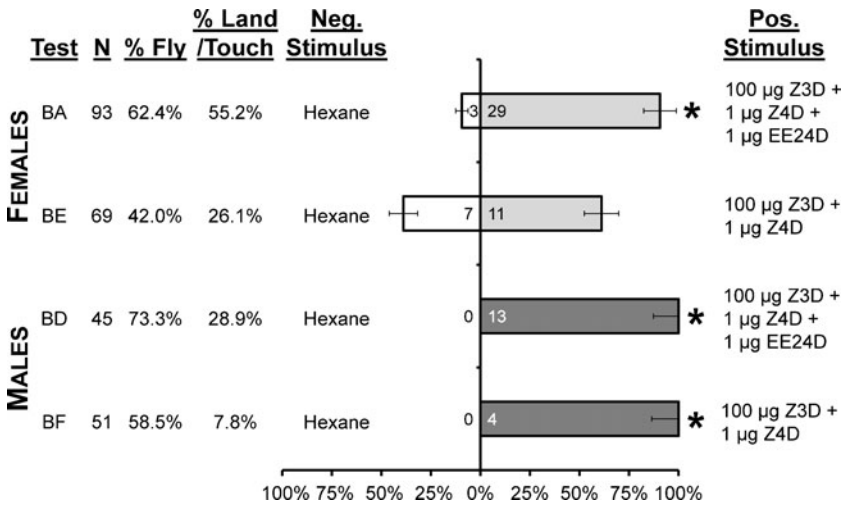

Fig. 5 Wind tunnel tests evaluated the attraction of female (*light gray bars*) and male (*dark gray bars*) *Sirex noctilio* to one of two 5-cm discs attached to lures with either the 2- or 3-component blends, or the hexane control (*white bars*). Displayed are the percent of males and females that flew upwind, the percent of those that flew upwind that made a choice by landing on or touching one of the two targets, and of those that made a choice, the percent that chose the hexane control vs. the lure with the synthetic blend. *Asterisk* indicates that choices were significant at α=0.05. Z3D=(*Z*)-3-decen-1-ol, Z4D=(*Z*)-4-decen-1-ol, and EE24D=(*E,E*)-2,4-decadienal. *Error bars* represent standard errors; *numbers* in bars represent number of individuals making each choice

The main component, (*Z*)-3-decen-1-ol, recently was described as a minor component of an aggregation pheromone of the banded alder borer, *Rosalia funebris* Mots. (Coleoptera: Cerambycidae) (Ray et al., 2009). The minor component (*E,E*)-2,4-decadienal was found in whole body washes of female *Orius insidiosus* (Say) (Hemiptera: Anthocoridae), but its behavioral function has not been determined. It was also found as an insect semiochemical in two species of stink bugs (Ho et al., 2003; Aldrich et al., 2007). The components also are emitted by various plants, especially those in the Orchidaceae family (Kaiser, 1993).

This study used only virgin males and females, but preliminary tests that included potentially mated females gave less consistent results. For this reason, we think this pheromone may be attractive only to virgin females. Males appear to have a 2-d maturation period in which they do not yet produce the pheromone, but they are still attracted to it during that time. It is unknown whether males have a similar maturation period for mating. Regardless, if this pheromone proves useful in trapping programs, it could potentially capture wasps before they have an opportunity to mate or oviposit.

Behavioral differences were noted between males and females in the wind tunnel when offered a choice between two 5-cm discs suspended 15 cm apart, one with the hexane control, and the other with either the 2-component blend or the 3-component blend. The 3-component lure worked better than the 2-component lure for both males and females in terms of activating upwind flight, frequency of making a choice, and correct choice being made. Females showed a strong preference for only the 3-component lure, and not the 2-component lure. Some females chose the hexane control, whereas none of the males chose it. With both lures, upwind flight was activated more often in males than in females, whereas landing or touching the target occurred more often in females than in males.

The part of the male genitalia that is displayed during excitation, the latomeres, are known in siricids to be used for grasping the female during copulation (Schulmeister, 2001; 2003), and this was observed in the laboratory during mating. The males also have long, robust hind legs that hook forward to hold onto the female while mating (MFC, pers. obs.). The fact that the latomeres are everted when no females are present suggests that they could have other roles as well. Males splayed their latomeres when rapidly walking or flying, or immediately before taking flight, suggesting they may provide stabilization. However, genitalia were not always everted during flight (MFC, pers. obs.), and usually were everted when other males or their odors were present, suggesting a possible role in emitting pheromone.

One challenge for management of *S. noctilio* in the northeastern United States is the lack of efficient detection tools, particularly effective trap designs. The lack of a strong attractant has confounded trapping efforts in the delineation of the expanding range of this invasive species. An attractive pheromone could improve detection capabilities for this species by facilitating research to improve trap designs, and allowing control measures to be focused in problem areas. Field studies are underway to examine the utility of this pheromone for surveillance applications.

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