Hawaii Invasive Species Council (HISC) Research and Technology Grant Program, Progress/Final Report

Project Title: Identifying Sex Pheromone Components of the Nettle Caterpillar, *Darna pallivitta* (Moore), to Facilitate Detection and Pheromone Disruption Control

Principal Investigators: Matthew S. Siderhurst[1], Eric B. Jang[1], Arnold H. Hara[2], Patrick Conant[3]

[1]US Pacific Basin Agric. Res. Center, USDA-ARS, P.O. Box 4459, Hilo, HI

[2]Dept. Plant Environ. Protect. Sci., CTAHR, University of Hawaii at Manoa, 461 West Lanikaula Street, Hilo, HI

[3] Hawaii Dept. Agric., 16 East Lanikaula St., Hilo, HI

Executive Summary: The nettle caterpillar, *Darna pallivitta* (Moore), is an invasive pest on the island of Hawai'i, causing defoliation of ornamental nursery stock and posing a human health hazard due to their painful sting. Wind tunnel and field tests with 2 day old moths revealed male behavioral responses to caged females consistent with a female released sex pheromone. Coupled gas chromatography-electroantennogram detection (GC-EAD) analysis of abdominal tip extracts revealed two male electroantennographically active compounds produced by female *D. pallivitta*. Mass spectral analysis and subsequent synthesis identified the active compounds as n-butyl (*E*)-7,9-decadienoate (major component) and ethyl (*E*)-7,9-decadienoate (minor component), both structurally similar to sex pheromone components previous reported from related *Darna* spp. Additionally, methyl (*E*)-7,9-decadienoate was identified from female abdominal extracts and a strong EAD response was elicited by the synthetic compound. n-Butyl (*E*)-7,9-decadienoate was the only component detected by solid phase microextraction (SPME) collections from single calling female moths, however the absence of the minor components may be a result of their lower abundance. Field trials showed significant attraction to all lures containing the major pheromone component while the minor components did not increase trap captures at the levels and ratios tested. Synthetic pheromone lures outperformed virgin moths as attractant baits and could be developed for monitoring *D. pallivitta* populations of the island of Hawai'i and detection on other Hawaiian islands.

Progress and completion of tasks 1-5, as outlined in the scope of services and restated below, are outlined in the following text.

1) Identify and synthesize or obtain sex pheromone components of the nettle caterpillar, Darna pallivitta (Moore), to allow rapid detection, pheromone disruption control, and monitor trapping of this pest species.

2) Collect or extract volatile pheromone material from D. pallivitta.

3) Identify and analyze electrophysiologically active compounds (using gas chromatography or electroantennagram detection) and structural determinations (using gas chromatography or mass spectrometry).

4) Synthesize pure compounds of interest by existing methodology, or obtain from commercial sources.

5) Conduct behavior bioassays with compounds of interest singly and in blends.

INTRODUCTION

Infestations of the nettle caterpillar were first discovered at a nursery in Panaewa, on the eastern side of the island of Hawai'i in September 2001 (Conant et al., 2002). *D. pallivitta* is known to occur in Southeast Asia where it feeds on palms (coconut and areca) and grasses (Cock et al., 1987) and was possibly introduced to Hawai'i with a shipment of *Rhapis* palm (*Rhapis excelsa*) seedlings from Taiwan (L. Nakahara, personal communication). Initial attempts to contain the *D. pallivitta* outbreak were not successful and the moth is now found across a large part of eastern Hawai'i. This nettle caterpillar has also been reported as an invasive species in Japan in 1997 (Tominaga, 1999; Yoshimoto, 1997). Transportation of contaminated nursery stock is a potential source of outbreaks both in unaffected areas of Hawai'i and on other islands.

D. pallivitta caterpillars have a wide host range, feeding on many agricultural crops, including coffee and macadamia, as well as landscape plants thereby threatening a nursery industry valued at $97.7 million (National Agricultural Statistics Service, 2004). Particularly susceptible to damage are palms, the single most valuable floriculture/nursery crop in Hawai'i, and dracaenas, which were worth a combined $12.9 million in 2003 (National Agricultural Statistics Service, 2004).

Additionally, the caterpillar constitutes a human health hazard due to the painful sting which results from contact with its spines. Current detection of the nettle caterpillar, *D. pallivitta,* relies on light trapping and visual surveys, techniques that are less specific, slower and more time consuming than pheromone trapping. Identification and synthesis of a pheromone lure for *D. pallivitta* would not only allow rapid detection of this irritant-causing/economically-important invasive pest, but also may provide control options such as pheromone mating disruption.

Pheromone component investigation has been undertaken with the heterogeneric moths *D. trima* and *D. bradleyi* (Sasaerila et al., 2000b). Both species show attraction to binary mixtures of (*E*)-7,9-decadienoates, a novel class of molecules used by Lepidoptera (Sasaerila et al., 2000b). Herein we report the results of GC-EAD and GC/MS analyses, synthesis and field testing of pheromone components for *D. pallivitta*.

MATERIALS AND METHODS

Insects. Caterpillars were collected from grasses on the USDA-ARS-PBARC facility, Hilo, HI and from Keaau, HI. Caterpillars were reared on cut Hawaiian ti (*Cordyline fruticosa* (L.)) and sorghum (*Sorghum* sp.) leaves in plastic rectangular containers (Rubbermaid, Fairlawn, Ohio). Cocoons were removed and placed in individual covered plastic cups. Additional

cocoons were obtained from the laboratory colony maintained on sorghum by the Hawai'i Department of Agriculture, Hilo, HI. Following eclosure, moths were sorted by sex and held in different chambers. Both cocoons and moths were held at ~24 ^{o}C on a 12 h:12 h light/dark cycle.

Volatile sampling. Female moths, 2-3 days old, were snap-frozen at -70 ^{o}C while exhibiting calling behavior 2-3 hours following the onset of scotophase. Abdominal tips including pheromone glands were subsequently excised and extracted for 5 minutes in hexane. Hexane extracts were then transferred into conical glass vial inserts and concentrated under a purified nitrogen stream. Single moth pheromone headspace sampling was carried out using a solid phase microextraction (SPME) fiber (polydimethylsiloxane (PDMS): 100 μm film, Supelco, Bellefonte, PA) introduce for 30 min. into a 1 ml glass vial containing a 2-3 day old calling female.

Instrumentation. Male electroantennographic responses to female abdominal extracts were recorded using an Agilent Technologies 6890 gas chromatograph (Palo Alto, CA) coupled to a Syntech electroantennogram detector system (Hilversum, Netherlands). The GC was equipped with an HP-5 column (30 m x 0.25 mm ID 0.25 μm film thickness) with helium as carrier gas (2.3 ml/min.) and makeup gas (10 ml/min), which were combined with a Y-type connector. A Graphpack-3D/2 flow splitter was attached to the base of the connector and the effluent was split 1:1 between the flame ionization detector (FID) and the EAD via a heated transfer line (250 ^{o}C). The injector, in splitless mode, and FID were held at 250 ^{o}C and 275 ^{o}C, respectively. The oven temperature program began at 80 °C for 1 min, then ramped at 10 °C/min to 240 °C, and was held for 13 min. Whole moth heads or excised antennae were secured with electrode gel (Spectra 360, Parker Laboratories, Inc., Fairfield, NJ) between the electrodes of a

Syntech EAG probe antenna holder. Humidified air was passed over the antennal prep and acted as a carrier for effluent from the EAD transfer line. The signal generated by the EAD was passed through a Syntech NL 1200 high-impedance amplifier and analyzed with the FID signal using Syntech GC-EAD2000 software version 2.5.

GC/MS analysis was performed on a Agilent Technologies 6890N gas chromatograph interfaced to a Hewlett-Packard 5973 Mass Selective Detector equipped with either an HP-5MS or DB-255MS column (both 30 m x 0.25 mm ID 0.25 μm film thickness). The temperature program used was 80 ^{o}C to 220 ^{o}C at 10 ^{o}C/min with a 1 min start delay with the injector temperature set at 250 ^{o}C using helium as a carrier gas (1.1 ml/min.).

^{1}H, ^{13}C, and COSY NMR spectra were obtained with a Varian Unity Inova 500 spectrometer equipped with a Varian 5 mm double resonance gradient probe. High resolution electron impact mass spectra (HREI MS) were obtained with a dual sector VG Analytical 70SE mass spectrometer run in EI mode with perfluorokerosine (PFK) used for reference.

Syntheses. Preparation of (*E*)-7,9-decadienoates was accomplished by modifications to the synthetic methods of Yamada et al. (1986) and Sasaerila et al. (2000b) (Fig. 1). *O*-tetrahydropyano-6-iodo-1-hexanol (**2**) was prepared by the monoprotection of 1,6-hexanediol (**1**) (Sigma-Aldrich, Saint Louis, Missouri) (Cossé et al., 2001) and subsequent iodination. Alkylation of 3-sulfolene (Fluka, Buchs, Switzerland) resulted in the sulfolene adduction **3** which was subsequently heated to induce extrusion of SO_2. The resulting diene was easily deprotected with *p*-toluenesulfonic acid monohydrate (TsOH·H2O) to give the free alcohol which was oxidized with pyridinium dichromate (PDC) to the acid (**4**). N,N′-

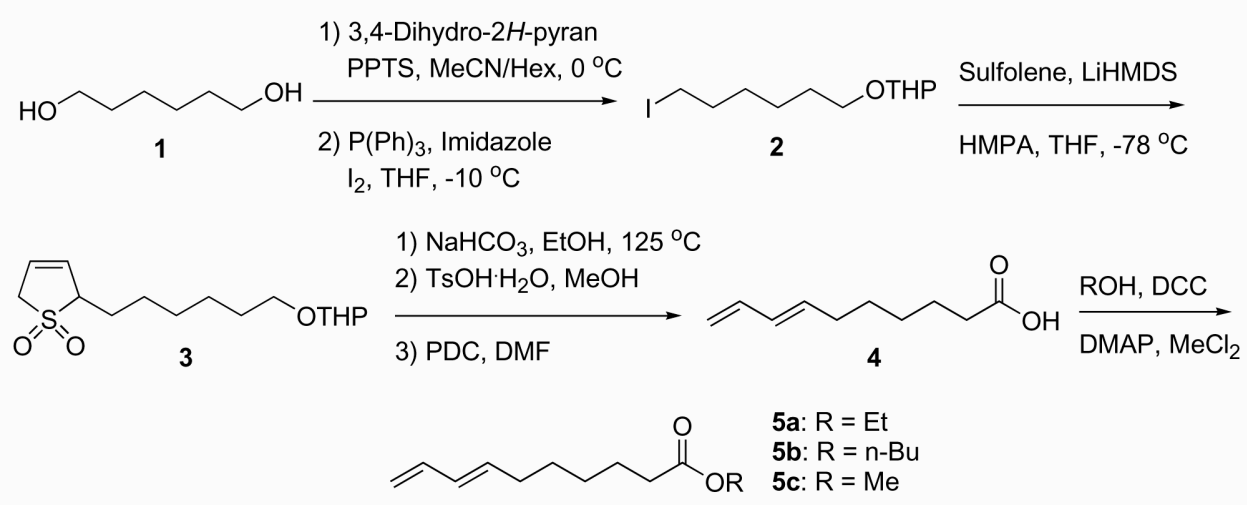

Figure 1. Synthetic scheme for alkyl (*E*)-7,9-decadienoates identified from *D. pallivitta* abdominal tip extracts based on Yamada et al. (1986) and Sasaerila et al. (2000b).

Dicyclohexylcarbodiimide (DCC) coupling of **4** with the appropriate alcohols, ethanol, n-butanol and methanol respectively, yielded the corresponding ester **5a-c**. Ethyl (*E*)-7,9-decadienoate (**5a**, Et *E*7,9-10:acid): clear viscous liquid; GC/MS (EI, 70 eV) *m/z* (rel. int.) 196 $[M]^+$ (14), 150 (45), 135 (19), 121 (20), 108 (51), 94 (36), 79 (64), 67 (100), 54 (48). n-Butyl (*E*)-7,9-decadienoate (**5b**, n-Bu *E*7,9-10:acid): clear viscous liquid; ^{1}H NMR (CH_3Cl, 300 MHz) δ 1.11 (3H, t, H-14), 1.47-1.60 (6H, m, Hz H-4, 5 & 13), 1.78 (4H, m, H-3 & 12), 2.25 (2H, dt, H-6), 2.45 (2H, t, H-2), 4.24 (2H, t, H-11), 5.12 (1H, d, J = 10 Hz H-10_{cis}), 5.25 (1H, d, J = 17 Hz H-10_{trans}), 5.85 (1H, dt, H-7), 6.21 (1H, d, H-8) and 6.47 (1H, dt, H-9); ^{13}C NMR (CH_3Cl, 300 MHz) δ 173.8 (C-1), 137.2 (C-9), 135.0 (C-7), 131.0 (C-8), 64.1 (C-11), 37.0, 34.2, 32.2, 30.6, 28.6, 24.8, 19.1 and 13.6 (C-14); HREI MS *m/z* 224.1810 $[M]^+$ (calcd. for $C_{14}H_{24}O_2$, 224.17763); GC/MS (EI, 70 eV) *m/z* (rel. int.) 224 $[M]^+$ (12), 150 (63), 135 (19), 121 (25), 108 (83), 93 (29), 81 (64), 67 (100), 54 (44). Methyl (*E*)-7,9-decadienoate (**5c**, Me *E*7,9-10:acid): clear viscous liquid; GC/MS (EI, 70 eV) *m/z* (rel. int.) 182 $[M]^+$ (26), 150 (37), 135 (15), 121 (17), 108 (50), 93 (36), 79 (67), 67 (100), 54 (61). A sample of crude (*E*)-7,9-decadienoic acid, used for initial structural confirmation, was obtain from Regine Gries at Simon Fraser University. Candidate esters were prepared by Fischer esterification by refluxing the crude acid with Dowex 50W cation exchange resin in the appropriate alcohol.

Bioassays. A laboratory flight tunnel, was used to investigate male responses to calling females and determine the time of calling. The glass/metal flight tunnel consisted of a 0.9 m x 0.9 m x 2.8 m rectangular glass arena equipped with inlet and exit fans, which produced a laminar flow of air (Jang et al., 1997). Assays were conducted as two-choice tests with ten 2-3 day old females held in a cylindrical screen cage (~1 L volume) containing several artificial leaves being run against a similar empty cage. For each assay, 5 males were released from the

downwind end of the flight tunnel. All assays were run between 1800-2400 hrs, at a temperature of 26-28 °C, under red light.

Field Experiments. Initial field tests to determine if females produced a sex pheromone were carried out with live, 2-3 day old, virgin female moths placed in Jackson traps (Delta type trap; sticky surface area ~130 cm). Female moths were confined to 3 x 3 cm conical screen cages containing a sugar-soaked cotton wick. Traps were monitored daily and female moths were replaced every 2-3 days.

Field trials 1 and 2 were conducted on the University of Hawai'i, Manoa, Waiakea Research Station, Hilo, HI, during October 2005. Test lure compounds were applied in CH_2Cl_2 to red rubber septa and placed on the sticky inserts from Jackson traps. Traps were randomized and hung ~1.5 m above the ground at 10 m intervals along fencerows boarding unmowed grass fields. Sticky inserts were removed daily, with the lure being transferred to the new insert, and the number of moths captured was recorded. Field trail 1 tested all component combinations at the ratios detected in moth extracts. Field trial 2 tested a dose response of n-butyl (*E*)-7,9-decadienoate from 2.5 µg to 2.5 mg. This experiment also included live, 2-3 day old, virgin female moths, which were replaced daily, as a positive control.

Data Analysis. Field data was transformed by $\sqrt{(x + 0.5)}$ to normalized distribution and was subsequently analyzed using ANOVA. Mean comparisons were carried out using the REGWQ test (SAS, 2000). All analyses of significance were made at the $P < 0.05$ level of significance.

RESULTS

Wind tunnel experiments showed male moths responding to caged females between 2 and 4 hours after the onset of scotophase. This coincided with observations of female calling

behavior. Male behaviors observed in the wind tunnel were indicative of the characteristic lepidopteran response to female produced pheromone including upwind directed flight and alighting on screen cages containing calling females. Field trapping with virgin females resulted in substantial captures of male moths additionally supporting the existence of a sex pheromone. GC/EAD analysis of female abdominal tip extracts revealed consist male electroantennographic activity to two compounds A (minor component) and B (major component) (Figure 2). GC/MS (EI, 70 eV) revealed structural similarities between the active compounds and with alkyl (*E*)-7,9-decadienoates previously reported for *Darna* spp. (Sasaerila et al., 2000b). The mass ions for A and B, which were of strong relative intensity, were *m/z* 196 and 224 respectively, suggesting an ethyl ester and a butyl ester isomer of decadienoic acid (Figure 3). Extracted ion analysis (Enhanced ChemStation, Agilent Technologies) revealed the presences of an addition compound (C) with a *m/z* 150 fragment and mass spectra which closely matched methyl (*E*)-7,9-decadienoate (Sasaerila et al., 2000b) (Table 1).

Fischer esterification of crude (*E*)-7,9-decadienoic acid with selected alcohols, furnished sufficient amounts of methyl, ethyl and the four possible butyl isomer ester for GC/MS mass spectra and retention time comparisons with moth extracts. These comparisons further suggested the EAD active compounds A and B were ethyl and n-butyl (*E*)-7,9-decadienoate while C was the methyl ester. Total synthesis (Figure 1) yielded the three (*E*)-7,9-decadienoates of interest (~250 mg total mass) and further confirmed the above identifications. Synthetic n-Bu *E*7,9-10:acid was further analyzed by 1-D and 2-D ^{1}H NMR, ^{13}C NMR, and HREI MS to confirm its structure. Due to the small amounts of synthetic material produced, Et and Me *E*7,9-10:acid were not subjected to NMR or HREI MS analysis.

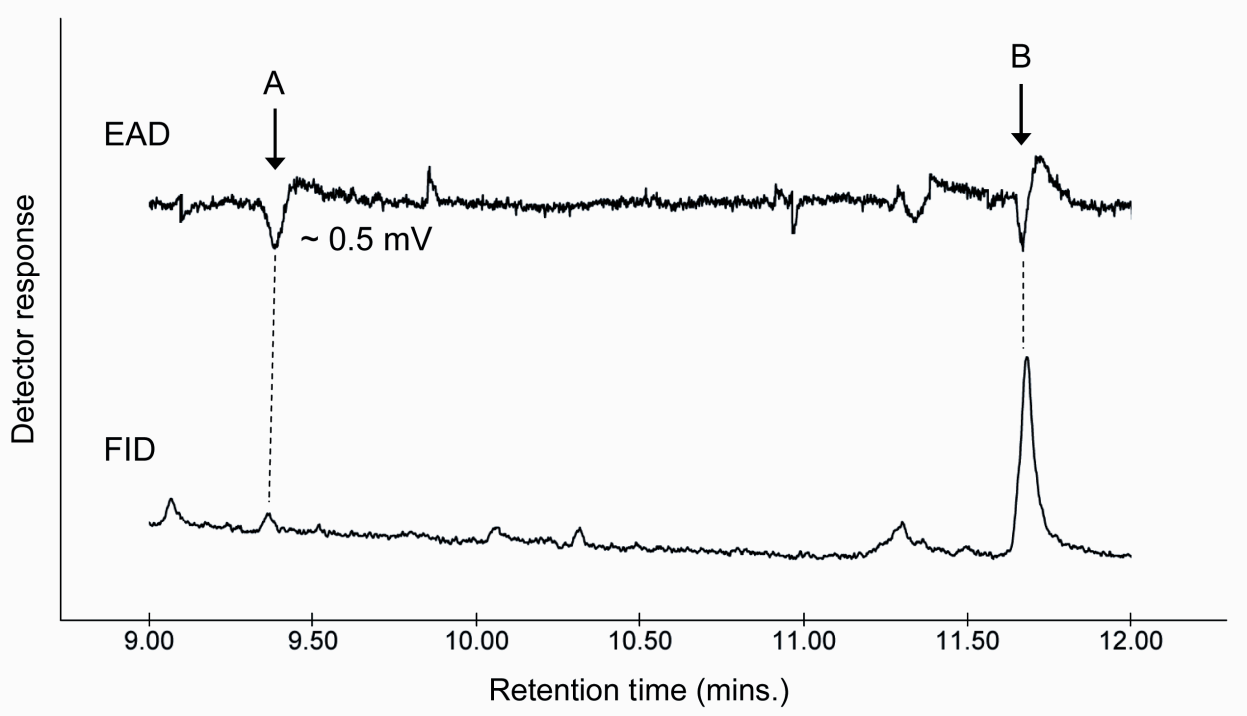

Figure 2. Electroantennogram detector (EAD: male moth antenna) and flame ionization detector (FID) responses to female abdominal tip extracts.

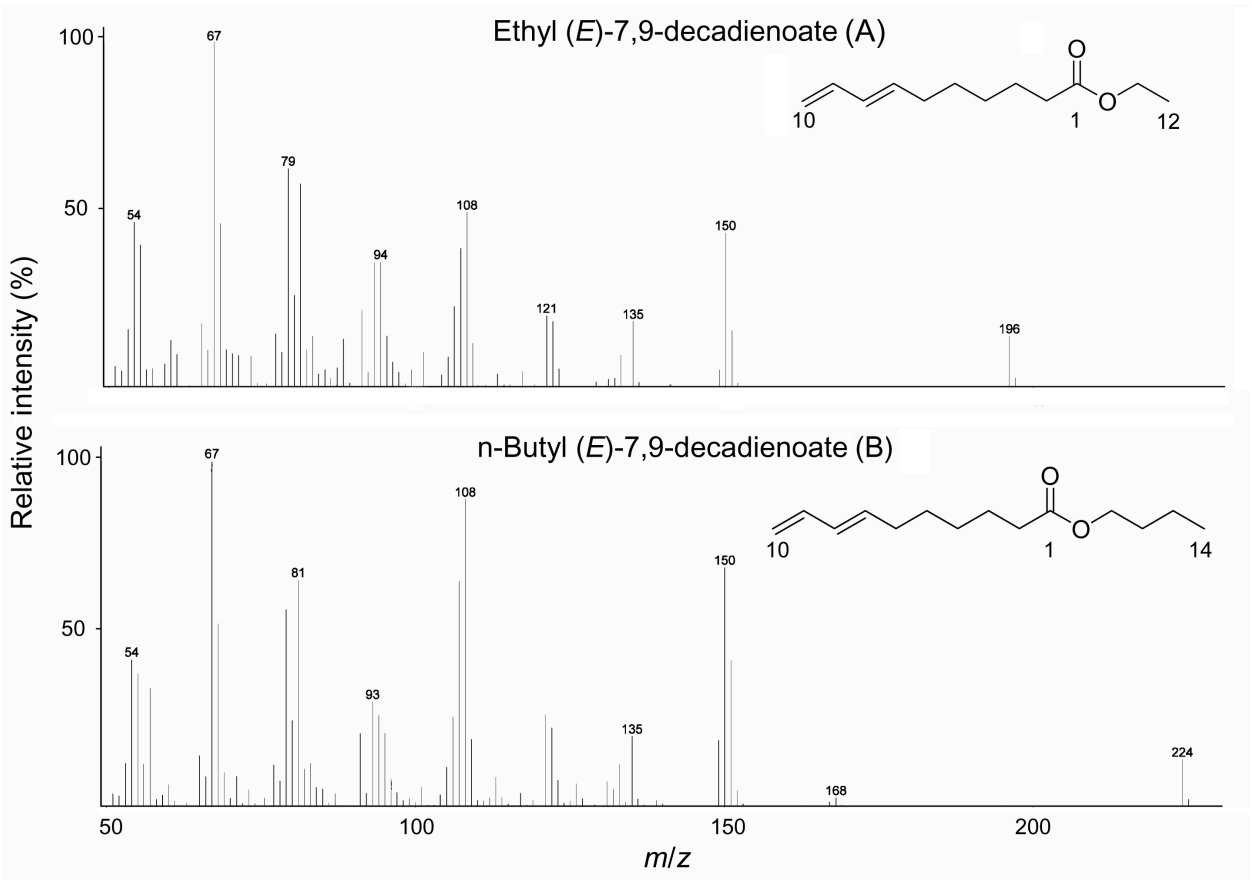

Figure 3. Electron impact mass spectra (EIMS; 70 eV) of EAD active compounds **A** (ethyl (*E*)-7,9-decadienoate) and **B** (n-butyl (*E*)-7,9-decadienoate). Number system indicated for each structure.

Table 1. Retention times of synthetic and moth extracted alkyl (*E*)-7,9-decadienoates pheromone components on non-polar and polar GC columns.

	Retention time (mins)			
	Moth extract		Synthetic	
	HP-5MS	DB-255MS	HP-5MS	DB-255MS
Me E7-9-10:acid	8.63	9.27	8.63	9.28
Et E7-9-10:acid	9.55	9.89	9.52	9.88
n-Bu E7-9-10:acid	11.89	11.89	11.90	11.87

Single moth pheromone headspace sampling, with a SPME fiber collection and subsequent GC/MS analysis, was only able to detect the presence of n-Bu *E*7,9-10:acid released by calling females.

Field trial 1, with all component combinations at moth extract ratios, showed significant male captures in all treatments baited with n-Bu *E*7,9-10:acid (Anova df = 5; F = 26.03; $P < 0.001$) (Figure 4). The presence or absence of Et and Me *E*7,9-10:acid did not affect the number of moths captured indicating that these compounds are not behaviorally active at the levels and/or ratios tested. Field trial 2, which tested the dose response of the most active treatment from the previous experiment, showed an increase in trap captures with greater amounts of n-bu *E*7,9-10:acid (Anova df = 7; F = 42.98; $P < 0.001$) (Figure 4). Caged, 2-3 day old, virgin females captured less than half as many male moths as the 2.5 mg n-Bu *E*7,9-10:acid lure, and was not significantly different from the 25 and 250 μg lures.

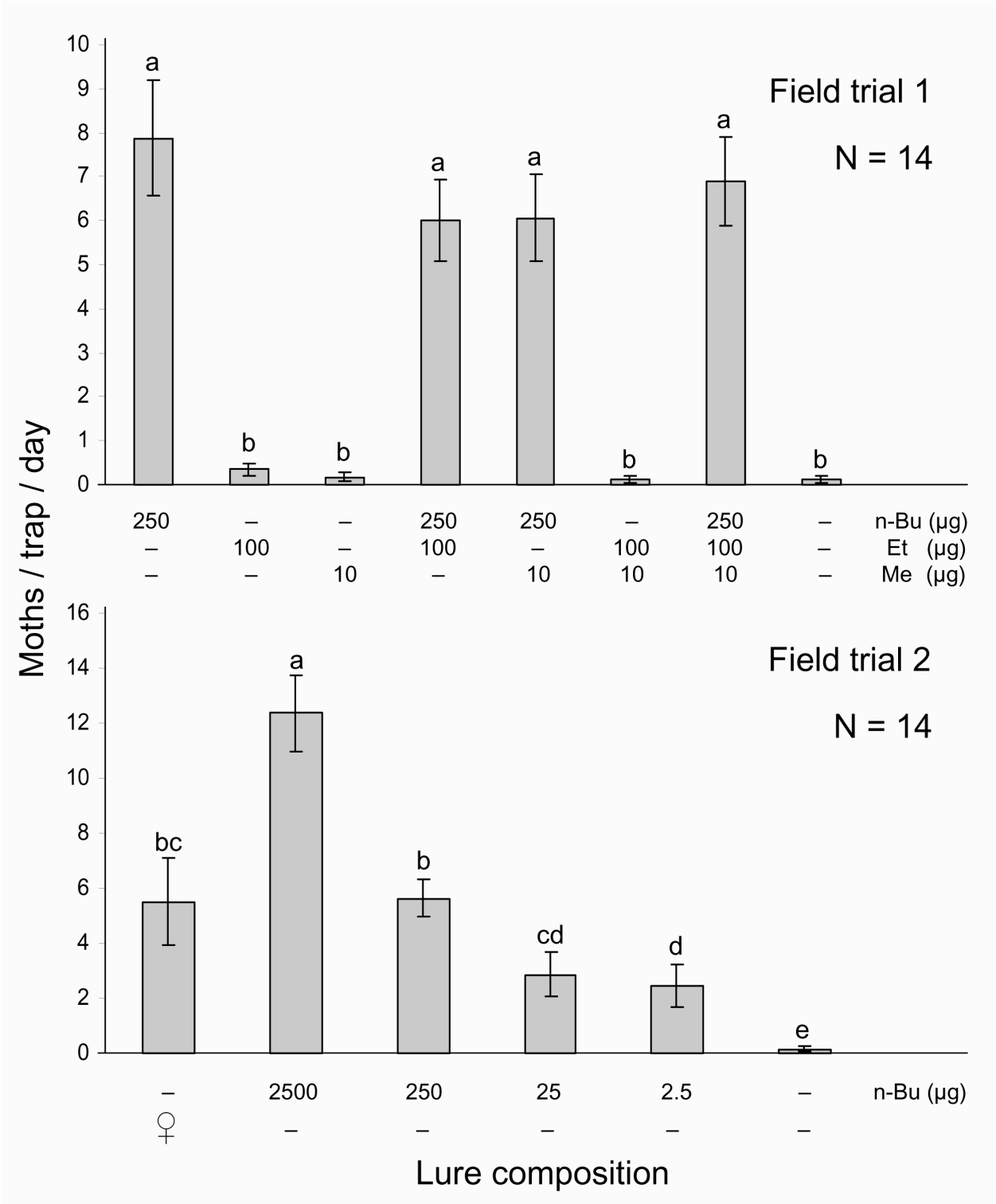

Figure 4. Male moths captured (mean ± SE) in sticky Jackson traps in field trials conducts on the UH Manoa, Waiakea Research Station, Hilo, HI. Field trail 1 tested all component combinations at the ratios detected in moth extracts. Field trial 2 tested a dose response of n-butyl (*E*)-7,9-decadienoate with live, virgin, female moths included as a positive control. Different letters indicate significant difference at $P < 0.05$; REGWQ (SAS, 2000).

DISCUSSION

Comparison of the EAD active compounds isolated from *D. pallivitta* show a high degree of structural conservation with pheromone compounds isolated from heterogeneric species. Alkyl (*E*)-7,9-decadienoates as pheromone components are so far limited to *Darna* spp. and are the only compounds used within the genus. Of the gland extracted (*E*)-7,9-decadienoates, the ethyl and n-butyl esters constituent new reports of moth electrophysiologically active compounds while the methyl ester is previously known as part of the *D. bradleyi* pheromone blend.

The observation that only n-Bu *E*7,9-10:acid baited traps caught significant numbers of moths, at the ratios and concentrations tested, and that it was the only compound detected from calling females, lead to the assignment of n-Bu *E*7,9-10:acid as the predominant component of the *D. pallivitta* pheromone. This is in contrast to not only heterogeneric spp. but other limacodids, which all employ blends of two synergistic compounds (2000b; 2000a; Sasaerila et al., 1997). Traps baited only with synthetic n-Bu *E*7,9-10:acid pheromone lure outperformed virgin moths suggesting that this compound is a good candidate to be developed for monitoring *D. pallivitta* populations of the island of Hawai'i and detection on the other Hawaiian islands.

ACKNOWLEDGEMENTS

We would like to thank Lori Carvalho, Janice Nagata and Esther Schneider for their assistance in conducting bioassays, electrophysiological recordings, compound synthesis and moth rearing. Louis Bjostad provided very helpful advice on methods and approach. We are also grateful to Regine Gries (Simon Fraser University) for providing *E*7,9-10:acid and Walt Niemczura (University of Hawai'i) for conducting NMR and HREI experiments.

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Progress and completion of task 6, as outlined in the scope of services and restated below, are outlined in the following text.

6) Disseminate findings to action agencies and the scientific community through submission of journal articles, presentations at conferences, and personal contacts to insure further development of the technology for detection and control applications.

Findings have been disseminated through the following:

- Oral and poster presentations: CTAHR Pest Control Field Day (14 October 2005), Asia-Pacific Congress of Entomology (20 October 2005), MIDPAC tradeshow (26 October 2005), International Chemoreception Workshop on Insects (20-24 February 2005) and ESA Pacific Branch Meeting (5-8 March 2006).

- Manuscript in preparation: Siderhurst M. S., E.B. Jang, A.H. Hara, P. Conant. 2005. n-Butyl (*E*)-7,9-decadienoate: Sex pheromone attractant for the nettle moth, *Darna pallivitta* **(**Moore)

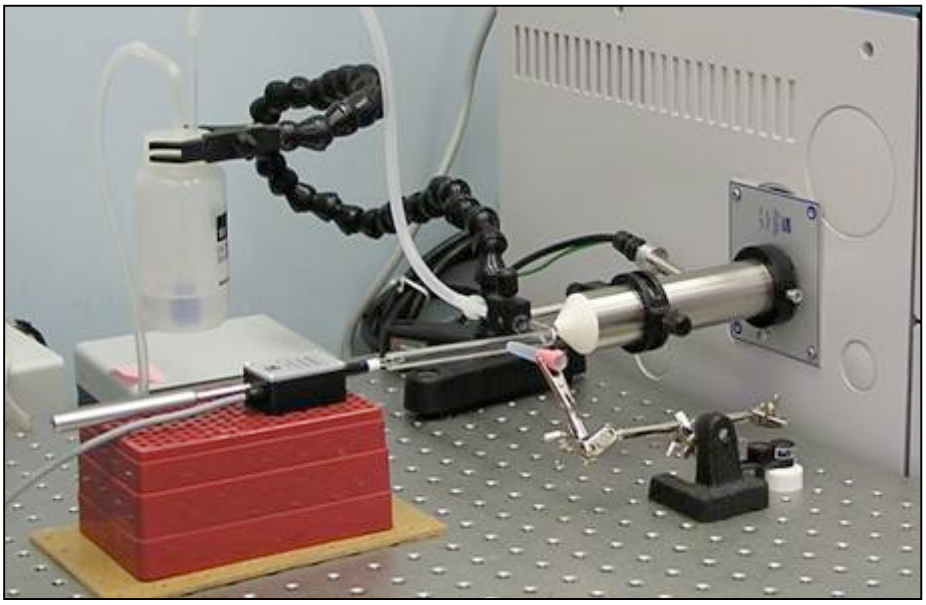
Progress and completion of task 7, as outlined in the scope of services and restated below, are outlined in the following text.

7) Plan further research with cooperators to implement findings in monitoring and control efforts.

Submitted and received a second grant to HISC entitled, "Detection, Control and Phenology of the Nettle Caterpillar, *Darna pallivitta* (Moore): Applications of a Pheromone Lure", which will further address the biology of this pest species and attempt to provide sufficient quantities of the pheromone lure to promote up-take of this technology in the community. Ongoing discussion with state and university collaborators.

Digital photographs:

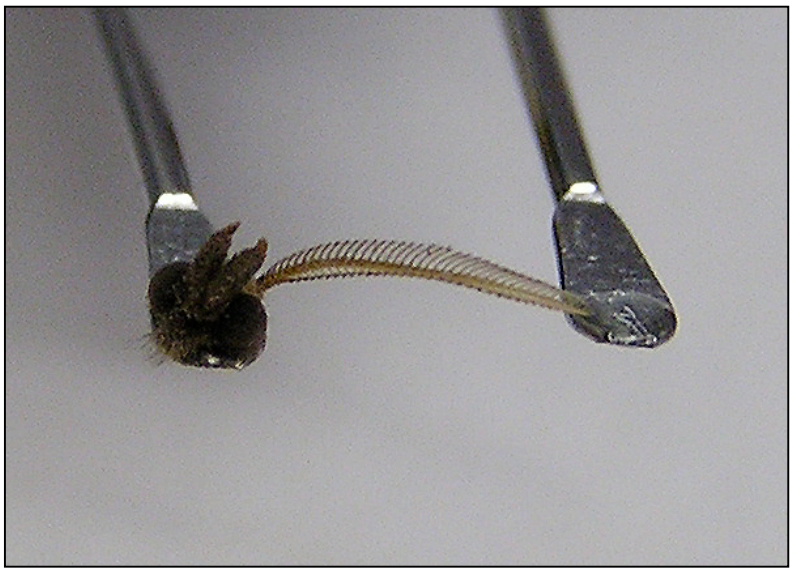
Male moths captured with by a trap baited with a virgin female.



GC/EAD setup.

Trap line used to evaluate different lure compositions.

Sample single night capture from Puna, HI.



Male moth head mounted between EAD electrodes.

Cages containing virgin females moths in wind tunnel.