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EFFECTS OF SOLDIER-DERIVED TERPENES ON SOLDIER CASTE DIFFERENTIATION IN THE TERMITE *Reticulitermes flavipes*

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Termite caste-regulatory terpenes

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Abstract - Primer pheromones play key roles in regulating division of labor, which is one of the most fundamental and defining aspects of insect sociality. Primer pheromones are chemical messengers that transmit hormone-like messages among colony members; in recipients these messages can either induce or suppress phenotypic caste differentiation. Here, we investigated soldier-caste-derived chemicals as possible primer pheromones in the lower termite *Reticulitermes flavipes*, a species for which no primer pheromones have yet been identified. We determined that soldier head extracts (SHE), when provided to totipotent workers along with the insect morphogenetic juvenile hormone (JH), significantly enhanced soldier caste differentiation. When applied alone, however, SHE had no impacts on caste differentiation, survivorship, or any other aspect of worker biology. These findings support that soldier-derived chemicals serve as primer pheromones which enhance the action of the endogenous morphogenetic hormone JH. Thus, SHE chemicals apparently have no effect when received under natural conditions by non-receptive individuals with presumably low JH titers. Gas chromatography-mass spectrometry analysis identified two terpenes as the most plentiful components of *R. flavipes* SHE. Through GC-MS and NMR analyses, these terpenes were identified as γ-cadinene and its corresponding aldehyde, γ-cadinenal. Validative bioassays with commercially available cadinene confirmed its activity. However, several other previously identified terpenes were also significantly active. These findings reveal a novel primer pheromone-like function for soldier-derived terpenes in termites, and further suggest convergent evolution of terpene functions in enhancing JH-dependent soldier caste differentiation.

Key Words- Termite; soldier; primer pheromone; juvenile hormone; terpene.

INTRODUCTION

Social insect castes are groups of phenotypically, morphologically and behaviorally distinct individuals that cooperate to perform colony tasks (Wilson, 1971; Miura, 2004). Caste differentiation plays an important and necessary role in creating an effective division of labor. It is imperative that colonies find ways to regulate caste differentiation within this system. Improper regulation could result in the over-abundance or absence of specific castes, making colony tasks such as food acquisition, grooming, defense, and reproduction inefficient or even impossible.

Polyphenisms are alternative morphological phenotypes that differentiate in response to environmental conditions (Nijhout, 2003). Termites use polyphenism to produce different castes that perform complementary roles within the colony (Miura, 2004). Termite colonies are made up of three distinct castes that include workers/pseudergates, soldiers, and reproductives. Only soldiers and reproductives are considered adults in lower termites, while all castes can be adults in higher termites. Termite caste differentiation can proceed along two routes; the imaginal (winged) or the apterous (wingless) route. The first developmental branch point occurs when larvae differentiate into either workers or nymphs after the second instar (Buchli, 1958, Lainé and Wright, 2003). Workers can: (1) undergo status quo worker-to-worker molts, (2) differentiate into presoldiers (immediately followed by soldier differentiation) or (3) differentiate into apterous and eyeless third-form reproductives, or "ergatoid neotenics". Nymphs can either; (1) regress into worker-like pseudergates, (2) differentiate into fully winged and eyed adult alates that disperse, mate, and become primary reproductives, or (3) differentiate into wingless and eyed non-dispersive second form reproductives, or "brachypterous neotenics" that serve as supplemental reproductives (Buchli, 1958; Lainé and Wright, 2003).

Caste polyphenism in social insects is distinct from solitary insects because multiple castes that perform non-overlapping tasks are present in colonies at the same time (Miura, 2004). Individuals in termite colonies with the same genetic background can

differentiate into alternate phenotypes depending on a number of intrinsic and extrinsic factors (Lenz, 1976; Greenberg and Tobe, 1984; Koshikawa et al., 2005; Scharf et al., 2007). One intrinsic factor is juvenile hormone (JH) (Scharf et al., 2003b; Park and Raina, 2004, 2005; Mao et al., 2005). Juvenile hormone is a morphogenetic hormone produced by a neurosecretory gland (the corpus allatum) that has a broad range of developmental and physiological effects (Wigglesworth, 1935; Schal et al., 1997; Truman and Riddiford, 1999; Gilbert et al., 2000; Truman et al., 2006). For example, in insects juvenile hormone plays a role in the control of larval/ nymphal development and metamorphosis, diapause, migratory behavior, wing length, seasonal development, reproduction, and caste determination (Hartfelder, 2000).

Primer pheromones are chemical messengers that are passed among individuals and trigger physiological responses in recipients (Wilson and Bossert, 1963). Primer pheromones are distinct from "releaser" pheromones, which elicit rapid behavioral responses in recipients (Vander Meer et al., 1998). Two examples of releaser pheromones in termites are the trail pheromone (Z,Z,E)-3,6,8-dodecatrien-1-ol (Matsumura, 1968) and the phagostimulatory pheromone hydroquinone (Reinhard et al., 2002). Three examples of primer pheromones from the honey bee are worker behavioral maturation inhibitory pheromone (ethyl oleate; Leoncini et al., 2004), brood pheromone (fatty acid esters; LeConte et al., 2006), and queen mandibular pheromone (5 carboxylate and aromatic components; Grozinger et al., 2007). Although no primer pheromones have been identified in termites, JH has been proposed as a possible termite primer pheromone (Henderson, 1998). Previous studies have shown that ectopic exposure of worker termites to JH III readily induces soldier caste differentiation (Howard and Haverty, 1979; Scharf et al., 2003b, 2005, 2007; Scharf et al., 2003b; Scharf et al., 2005; Zhou et al. 2006a,b, 2007Zhou et al., 2006a; Zhou et al., 2006b; Zhou et al., 2007), indicating that JH can act via exogenous exposure. Under natural conditions, high endogenous JH titers in worker termites cause differentiation into presoldiers, and then into soldiers (Park and Raina, 2004; Mao et al., 2005). Regardless of whether it acts exogenously as a primer pheromone, an endogenous hormone, or both, the role of JH in soldier development is

unique and in contrast to the immature "status quo" role of JH among insects (Henderson, 1998).

It has been hypothesized that termite soldiers may play a role in regulating worker differentiation to other caste phenotypes (Henderson, 1998). For example, JH titers in workers rise upon removal from the colony (Okot-Kotber et al., 1993; Mao et al., 2005), which can result in presoldier / soldier formation (Mao et al., 2005). However, if workers are held with soldiers, worker JH titers remain below threshold levels and presoldier formation is attenuated (Mao et al., 2005; Park and Raina, 2005). It has been theorized that soldiers can down-regulate worker JH titers by acting as a JH "sink" (Henderson, 1998; Mao et al., 2005) or by lifting some other primer pheromone's inhibition on worker differentiation (Park and Raina, 2004, 2005; Mao et al., 2005).

Previously, Lefeuve and Bordereau (1984) investigated live soldiers and the effects of methylene chloride (dichloromethane; DCM) soldier head extracts (SHE) on caste differentiation in the higher termite *Nasutitermes lujae*; they found that SHE inhibited worker-to-soldier differentiation. They further suggested that soldier termites may secrete an inhibitory pheromone that contributes to worker-soldier homeostasis in termite societies. Korb et al. (2003) also reported that DCM SHE inhibited soldier formation in the lower termite *Cryptotermes secundus*. Additionally, Okot-Kotber et al. (1991) also showed that soldier formation in *Reticulitermes flavipes* was reduced by DCM SHE when co-applied in combination with synthetic JH analogs. While these studies have verified primer pheromone-like effects for SHE, no *bona-fide* termite primer pheromones have yet been chemically identified. Thus, two important outstanding questions in termite research relate to whether or not caste-regulatory primer pheromones exist, and if so, what are their chemical structures and modes of action?

R. flavipes and its European synonym *R. santonensis* are common and economically destructive termites in the U.S. and Europe; thus, there is a need to define their chemical ecology with respect to cast regulation. The central objective of this study was to investigate chemical constituents of *R. flavipes* SHE as possible primer

pheromones. To meet this objective, we conducted studies to (1) investigate SHE effects on JH-dependent soldier caste differentiation, (2) identify SHE constituents, and (3) compare constituent activity with previously identified soldier head chemicals. Through these studies, we provide evidence supporting the idea that soldier-derived terpenes play roles as caste-regulatory primer pheromones in termites.

METHODS AND MATERIALS

Termites. *R. flavipes* colonies were collected from various locations on the University of Florida campus. Termites were brought back to the laboratory and held for at least 2 months before use. Laboratory colonies were maintained in darkness within sealed plastic boxes, at 22^{o}C. A total of 9 termite colonies were tested, all of which contained male and female neotenic reproductives. Termite workers were considered workers if they did not possess any sign of wing buds or distended abdomens. Termites were identified as *R. flavipes* from sequence of the 16S mitochondrial-ribosomal RNA gene, (Szalanski et al., 2003), gut fauna (Lewis and Forschler, 2004) and soldier morphology (Nutting, 1990).

Dish Assays. Dish assays were conducted at 27°C as described previously (Scharf et al., 2003b). Paired paper towel sandwiches were treated with respective control, JH III, and SHE treatments delivered in solvent (acetone). JH III (75% purity; Sigma; St. Louis, MO) was provided at 112.5 µg per dish in a volume of 200 µl acetone. This JH quantity was chosen based on its maximal efficacy and minimal mortality observed in previous concentration range studies (Scharf et al., 2003b). SHE was tested at several different quantities (see next section). After solvent evaporation, sandwiches were placed in 5 cm plastic Petri dishes and then received 150 µl of reverse osmosis water. Fifteen worker termites were placed in each dish. Every five days termites were counted, presoldier formation was noted, and deionized water was added if needed.

Solider Head Extracts. Soldier head extract (SHE) was prepared by collecting soldiers from lab colonies, removing their heads, and then by homogenizing the heads (~80-150 total, depending on the experiment) in acetone with a Tenbroeck glass homogenizer. SHE

was fractionated by passing it through a glass Pasteur pipette filled with approximately 250 mg of silica gel (60-200 mesh) on top of a glass wool plug. The eluting solvent in fractions consisted of 10 column volumes of the extraction solvent (acetone). The fractionated SHE was then brought to 50 ml in a volumetric flask.

SHE Concentration Response and Investigation of Colony Variation. SHE prepared in acetone was tested at multiple concentrations on three *R. flavipes* colonies (Colonies 7, 8 and 9). Seven different treatments were tested: control (300 µl acetone), JH III (200 µl acetone containing 112.5 µg JH III), SHE alone (4 head equivalents), and JH III plus a range of soldier head extract equivalents (0.5, 1, 2 and 4). Each treatment was replicated six times.

Gas Chromatography (GC) and Mass Spectrometry (MS). Thirty soldier and worker heads from two different colonies (colonies 5 and 7) were extracted as described above (acetone), in a volume of 2 ml and evaporated under N_2 to 400 µl. Samples were first analyzed by GC/MS (electron ionization, 70eV) to confirm the presence of the previously published predominant terpenoids, namely γ-cadinene and γ-cadinenal (Nelson et al., 2001), and then subsequently quantified using a 6890 gas chromatograph (Agilent; Santa Clara, CA) coupled to a flame ionization detector as described in full by Schmelz et al. (2001). We also examined pine wood extracts, prepared from the same "shim" wood used to provision lab colonies (seasoned and kiln-baked), to specifically test the hypothesis that SHE chemicals are produced in termites *de novo*. Fresh pine wood sawdust (1.26 g) was extracted and analyzed as described above for head extracts (acetone).

To quantify semiochemical levels found in individual soldier heads, five individual soldier heads were extracted in a similar manner as above. Individual extracts were in a final volume of 400 µl; an internal standard of 400 ng of nonyl acetate was added to each sample. Samples were then separated by GC. Peaks were analyzed and quantified by comparing to the nonyl acetate standard.

Nuclear Magnetic Resonance (NMR) analysis. NMR analyses were performed to accurately identify the cadinene chemicals from the soldier heads. The two main peaks of the SHE were separated using preparative GC and analyzed by NMR. Initial sample preparation of soldier head solvent extracts utilized vapor phase extraction at 80ºC on polymeric adsorbent traps, followed by dichloromethane elution to remove less volatile contaminants (Schmelz et al., 2004). Micropreparative gas chromatography (GC) was accomplished using an Agilent (Santa Clara CA) 6890 gas chromatograph (He carrier gas; 5.7 ml min^{-1}; cool on-column injector set to track oven) with an DB-1 column (30 m long, 530 µm i.d., 0.50 µm film thickness) with the temperature programmed from 35 °C (2 min hold) at 10 °C min^{-1} to 260 °C (hold for 5.5 min). Recovery of separated GC fractions followed from Heath and Dueben (1998) with slight modification. Specifically a glass press-fit splitter was used at the end of the DB-1 column, coupling a 0.5 m (150 µm i.d. fused silica) capillary to the flame ionization detector (FID) and a second 0.5 m (350 µm i.d. fused silica) capillary directed to the heated transfer line and chilled glass capillary for sample collection. Under these conditions, the two predominant soldier head sesquiterpenes eluted at 16.1 and 18.9 min. Authentic standards of γ-cadinene were similarly chromatographed, eluted at 16.1 min and recollected for NMR.

1-Dimensional and 2-dimensional NMR spectra were acquired at 20ºC with standard techniques using TopSpin® (version 2.1) software on a Bruker Avance-II-600 spectrometer equipped with a 1 mm high-temperature superconducting (HTS) CryoProbe (Brey et al. 2006). Solutions of the SHE γ-cadinene, ~ 10 µg/15 µl, the authentic γ-cadinene, ~ 25 µg/17 µl and of the SHE γ-cadinene aldehyde, ~ 50 µg/10 µl, were prepared in $CDCl_3$ (99.96 atom % D). These solutions were added via a 110 mm-needled 10 µl syringe to 1 mm O.D. x 0.73 mm I.D. x 100 mm long capillary NMR tubes (Norell, Inc.). The capillaries were then attached to an appropriate Bruker MATCH™ apparatus before being lowered into the NMR magnet for analysis. Proton spectra were acquired at 600.23 MHz using 45º pulses, 32768 complex points over an 11 ppm spectral width (SW) – corresponding to a 2.48 second acquisition time (AT), and a 3 second relaxation delay (RD). The ^1H data was processed by zero filling the FID's to 32768 real points before application of line broadening (LB) and Fourier transformation. An exponential LB value

of 0.4 Hz was used for integrated spectra, and a negative LB value of (–) 0.2 Hz was used for "peak picking." The ^{1}H chemical shift axis was referenced to $CHCl_3$, assigned to 7.26 ppm (Gottlieb et al. 1997). Abbreviations in ^{1}H spectra: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, J = apparent coupling constants in Hertz. 2-Dimensional $^{1}H/^{1}H$-COSY data sets (SW = 8 ppm, AT = 0.21 seconds, RD = 2 seconds, 2-8 transients) were acquired with Bruker's "cosygpqf" pulse sequence as 2048 complex points in the directly detected dimension (DD) and 512 increments in the indirect dimension (ID), and they were processed with sine-function apodization into 1024 x 1024 point spectra. Carbon-13 spectra were acquired at 150.93 MHz using 45º pulses, 65536 complex points over a 220 ppm SW – corresponding to a 0.98 second AT, and a 3 second RD. The ^{13}C FID's were Fourier transformed after zero filling to 65536 real points and applying an exponential LB value of 2 Hz. The ^{13}C chemical shift axis was referenced to $CDCl_3$, assigned to 77.16 ppm (Gottlieb et al. 1997). Multiplicity-edited 2-dimensional $^{1}H/^{13}C$-HSQC data sets (^{1}H SW = 8 ppm, ^{13}C SW = 170 ppm, AT = 0.14 seconds, RD = 2 seconds, 48-96 transients) were acquired with Bruker's "hsqcedetgpsisp2.2" pulse sequence as 1348 complex points in the DD dimension and 256 increments in the ID dimension, and they were apodized with cosine squared-functions into 2048 x 512 point spectra.

Previously Identified Chemicals. Past research (Zalkow et al., 1981; Bagnères et al., 1990; Nelson et al., 2001; Quintana et al., 2003) and our own GC-MS efforts (current report) have identified a number of chemicals from termite soldier heads. Chemicals (or close structural analogs) were tested individually in dish assays on a single *R. flavipes* colony (Colony 5). All treatments were applied at 50 µg/dish, with and without JH III (300 µl acetone containing 112.5 µg JH III). Individual chemical treatments were provided at a quantity equivalent to approximately 1/2 of the JH III dose in order to test *synergistic* effects on JH III-induced presoldier differentiation. This amount [50 µg/dish] approximates endogenous cadinene levels found in 25 soldier head equivalents, based on GC-MS analysis. Treatments were as follows: controls (300 µl acetone), SHE alone (4 head equivalents in acetone), α-humulene (CAS: 6753-98-6, Fluka, Sigma Aldrich, St. Louis, MO), β -farnesene (CAS:18794-84-8, Bedoukian, Danbury, CT), cadinene (CAS:

29350-73-0**,** Vigon International, East Stroudsburg, PA), geranyl linalool (CAS: 1113-21-9, Acros, New Jersey, NJ), linalool (CAS: 78-70-6, Aldrich), farnesol (CAS: 4602-84-0, Bedoukian), (+)β-pinene oxide (CAS: 6931-54-0**,** Acros), limonene (CAS: 5989-27-5, Aldrich), nootkatone (CAS: 4674-50-4, Bedoukian), nerolidol (CAS: 7212-44-4, Bedoukian), α-pinene (CAS: 80-56-8, Acros), and geranylgeraniol (CAS: 24034-73-9**,** Fluka, Sigma Aldrich). Control treatments included acetone, JH III alone, SHE alone, JH III+SHE. All SHE was prepared in acetone. Each treatment was replicated three times.

Statistical Analyses. In all experiments the number and caste of each termite in each dish was counted every five days. The percentage of presoldiers formed out of the total number of workers put into each assay was used in statistical analyses (Scharf et al., 2003b, 2005; Zhou et al., 2006a,b) . Data were first analyzed for normality using the *Levene* test. If the data were not normal, the data were transformed to ranked averages and means separated using the *Tukey-Kramer* test ($p<0.05$). For bioassays with previously-identified soldier chemicals, ranked averages were separated using a *LSD Student t-test* ($p<0.05$).

RESULTS

SHE Concentration Response. Three colonies were examined in SHE dose-response bioassays using SHE prepared in acetone (**Fig. 1**). Two of the three colonies responded similarly, but one colony (colony-9) responded slightly differently, which led to a significant *colony* effect in the ANOVA (df=2,117, F=4.788, p=0.01). Nonetheless, a pooled dose-response analysis of the three colonies was conducted. Presoldier induction significantly increased when termite workers were co-exposed to SHE and JH III, as compared to treatments of JH III alone ($p<0.05$). Controls treated with either acetone or SHE alone resulted in no presoldier formation. Presoldiers first appeared between days 10 and 15, and reached maximum levels by day 25 in all SHE + JH III and JH III-alone treatments. This analysis verifies that SHE does indeed cause a significant increase in presoldier formation when combined with JH III; however, this effect is not significantly dose-dependent in the range of 0.5 - 4 head equivalents (df= 6,117, F= 32.32, p<0.0001).

GC- MS and NMR Analysis. GC-FID analyses of soldier head extract identified two major sets of peaks (**Fig. 2**). Retention times, peak size, and GC-MS spectra of the two sets of peaks have similar profiles as Zalkow et al., (1981) and Nelson et al., (2001), who identified γ-cadinene and γ-cadinenal as major whole-head extract components (**Fig. S2**). The first peak, γ-cadinene, was identified by comparing its spectra with those in the literature, as well as by a gas-chromatographic comparison with the same sample. Additionally, comparison of the SHE γ-cadinene and of an authentic sample of γ-cadinene (kindly provided by Dr. Bartelt, USDA-ARS-NCAUR; Peoria, IL) by GC-MS analysis (EI, 70 eV) gave the same EI mass spectra and identical GC retention times. The mass spectrum and the ^{1}H (600 MHz) NMR spectrum of the SHE γ-cadinene were the same as those described for γ-cadinene by Quintana et al. (2003). Our analyses further confirmed that the SHE γ-cadinene and the authentic sample of γ-cadinene produced the same NMR spectra. That is, except for trace impurities in the natural sample, they gave identical 1-dimensional (^{1}H) and 2-dimensional (^{1}H/^{1}H-COSY and ^{1}H/^{13}C-HSQC) NMR spectra.

The corresponding γ-cadinene aldehyde (γ-cadinenal), assumed to arise from allylic oxidation of the olefinic methyl group of γ-cadinene, was identified by comparison of its ^{1}H NMR spectrum (see data below) and EI-mass spectrum (**Fig. S2**) to those reported by Kaiser and Lamparsky (1983). Since we observed some small differences between their 400 MHz ^{1}H spectrum and ours at 600 MHz, we also report the details of our ^{1}H NMR spectrum here, along with the fifteen chemical shifts for the ^{13}C NMR resonances of the SHE γ-cadinene aldehyde.

NMR results are as follows; additional data and structural information can be provided upon request. ^{1}H NMR (600 MHz, $CHCl_3$ = 7.26 ppm (Gottlieb et al. 1997)) δ 9.47 (s, 1 H), 6.91 s, 1H), 4.74 (“d”, J = 1.5, 1 H), 4.62 (“d”, J = 1.3, 1 H), 2.52-2.46 (m, 1 H), 2.44 (ddd, J = 2.9, 4.0, 13.0, 1 H), 2.26 (d septets, J = 3.3, 6.9, 1 H), 2.15-2.07 (multiplets, 2 H), 2.06 (broad dt, J = 4.5, 13.1, 1 H), 1.97-1.91 (t of “five-line patterns”, 1H), 1.90-1.84

(multiplets, 2 H), 1.50-1.41 (multiplet, 1 H), 1.42 (tt, J = 3.2, 11.6, 1 H), 1.21 (dq, J = 4.2, 12.8, 1 H), 0.99 (d, J = 6.9, 3 H), 0.82 (d, J = 6.9, 3 H).

^{13}C NMR (151 MHz, $CDCl_3$ = 77.16 ppm (Gottlieb et al. 1997)) δ 194.76, 151.78, 151.75, 141.77, 104.65, 46.37, 46.05, 44.07, 36.05, 26.69, 26.65, 24.45, 21.87, 21.57, 15.34.

The average amount of γ-cadinene and γ-cadinenal from soldiers was 1.44 ± 0.29 and 9.42 ± 1.75 µg, respectively. The amount of γ-cadinenal was significantly higher than the amount of γ-cadinene (df= 1,8, F=20.2864, p=0.0020). Although weakly abundant in the worker extracts, the γ-cadinene and γ-cadinenal were substantially more prevalent in the soldier heads (**Fig. 2**). Pine wood extracts prepared using an identical extraction method did not indicate any similarity to chemicals found in SHE (**Fig. 2**), supporting that cadinene and cadinenal are produced *de novo*.

Cadinene and Previously Described Soldier Chemicals Enhance JH-induced Presoldier Differentiation. Twelve previously identified soldier-derived chemicals were tested for their ability to induce presoldier formation in dish assays. All of these previously described chemicals (except nootkatone and nerolidol), when tested in combination with JH III, caused significant increases in presoldier differentiation relative to JH III alone. When tested without JH III, the soldier chemicals caused no presoldier differentiation (df= 26,63, F= 14.4633, p<0.0001) (**Fig. 3**). Similar to all previous assays, no presoldiers were observed in acetone controls, while high presoldier induction levels (~80%) were observed in SHE + JH III treatments. Treatments of JH III alone induced significantly lower presoldier levels (~20%), which are comparable to results of preceding experiments as presented above.

DISCUSSION

In previous research, termite soldier-produced chemicals have mostly been investigated as a taxonomic tool for species identification (Zalkow et al., 1981; Prestwich, 1983; Bagneres et al., 1990; Nelson et al., 2001; Quintana et al., 2003, Nelson etl al. 2008). Such research has identified a number of chemicals in soldier secretions, but little consideration has been given to roles of these chemicals in caste differentiation. The study presented here confirms the effects of *R. flavipes* SHE on JH-induced presoldier differentiation. Results from multiple bioassays on different colonies at different times of the year indicated that SHE synergistically increased worker to soldier morphogenesis when applied in combination with JH III. These findings support the idea that the soldier caste, in addition to playing a defensive role, also plays a part in caste regulation within termite society (Henderson, 1998).

Our study also supports previous research showing ectopic JH III treatments cause some workers to molt into presoldiers (and onto soldiers) (Scharf et al., 2003b, 2005, 2007; Zhou et al. 2006a,b). The JH III mediated worker–to-soldier molt is an atypical example of a JH III response when compared to other insect groups. In most insects, JH causes insects to remain as immature forms during a molt, while the absence of JH causes the insect to molt into an adult form. Thus, termites have apparently co-opted JH for a different function than other insect groups.

The combination of SHE with ectopic JH III treatments synergistically enhanced presoldier development relative to JH III alone, while SHE by itself caused no presoldier induction. This suggests that SHE probably does not contain significant quantities of JH. Preliminary thin layer chromatography separations of JH III and SHE showed no common bands (MRT unpublished), supporting the absence of JH III in SHE. Therefore, in our assays, we conclude that chemicals from soldier heads modulate the termite response to ectopically applied JH III, thereby enhancing JH III activity. We hypothesize that, endogenously, the synergistic effect of these SHE terpenes is manifest only in individuals with elevated JH titers.

The results from this study are in contrast with past reports concluding that soldiers and extracts from soldiers inhibit presoldier formation (Lefeuve and Bordereau, 1984; Okot-Kotber et al., 1991; Korb et al., 2003). There are several differences between the current and past research that could at least partially explain these discrepancies. First, Lefeuve and Bordereau (1984) exposed groups of 200 workers of the higher termite *Nasutitermes lujae* to one of three treatments that included nothing, live soldiers, or SHE extracted in DCM. Differences between this and our study include extraction solvent, termite species, and group size. Korb et al. (2003) tested the effect of precocene II, an allatectomizing agent, and SHE extracted in DCM on whole colonies of the lower termite, *Cryptotermes secundus*. Differences between our study and that of Korb et al. (2003) include solvent, termite species, and treatment size. Also, Korb et al. did not test precocene in combination with natural JH or SHE. Okot-Kotber et al. (1991) tested combinations of methoprene and SHE extracted in DCM on *R. flavipes* in a dish assay, similar to ours, and found that the combination resulted in less presoldier formation than treatments of methoprene alone. In unreported work, we found no difference between SHE extracted in DCM or acetone (see Table S1 and Fig. S1 included as supplementary online material), eliminating the effect of solvent. However, we used JH III in our study while Okot-Kotber et al. (1991) used the JH analog methoprene. Other factors that may explain some of the differences between our study and preceding studies may be colony conditions at the time of testing and the time of year at which testing was performed; e.g., responses to SHE and JH may vary among termite colonies, as well as within a colony over a year according to season.

While our results suggest components of *Reticulitermes* SHE function as primer pheromones, soldier secretions of other termite species have *bona fide* defensive functions. For example, *Coptotermes* soldiers produce latex to defend against predators (Prestwich, 1983, 1984; Abe et al., 2000). Our proposed primer pheromone function for *Reticulitermes* head chemicals, is supported by a study by Zalkow et al. (1981) who assayed a number of *R. flavipes* and *R. virginicus* soldier head chemicals against the native fire ant, *Solenopsis geminata*. Their results indicated the ants had not been sprayed

with an irritant or toxicant and that the soldier head chemicals have non-defensive functions.

No evidence was obtained in the present study to suggest that the chemicals are expelled from soldiers. One explanation for the soldiers having a large amount of putative primer pheromone in their heads is to serve as a recruiting mechanism after an individual soldier is killed. For example, if a soldier is killed when defending the colony, the chemicals acquired while disposing of the body may signal nestmate workers to differentiate into soldiers. Since workers also contained small amounts of cadinene and cadinenal, another possibility is that soldiers may absorb and sequester these compounds away from workers in order to suppress worker differentiation. For example, live soldiers suppress worker JH titers and inhibit presoldier formation (Park and Raina, 2004; Mao et al., 2005). Future research efforts will test hypotheses relating to impacts of live and dead soldiers in nestmate differentiation and terpene sequestration.

Of the soldier head terpenes identified in previous research, all but two significantly enhanced JH-induced presoldier formation when combined with JH III at a ratio of 1:2 (terpene:JH). When applied at the same concentration without JH III, however, none of the terpenes induced presoldier formation. This suggests that *Reticulitermes* have the ability to utilize an array of terpenes as cues to trigger soldier caste differentiation, provided that endogenous JH titers are above critical thresholds. Future research should determine what structural features of the terpenes are necessary for activity.

The regulation of termite caste differentiation is important in maintaining social structure and function, and therefore the disruption of termite caste differentiation / homeostasis may be able to be used as a control method. By using the termite's own chemistry (*i.e.*, soldier-derived terpenes), it may be possible to develop a specific termiticide that causes a large proportion of worker termites to molt into soldiers. Because soldier termites cannot feed themselves, this would likely cause the termite colony to starve or at least have a severe effect on the colony. For example, in our study

(unpublished results), mortality was greatest in replicates in which a high proportion of worker termites molted into presoldiers.

In summary, the findings presented here verify a role beyond defense for the soldier caste within termite societies, as initially proposed by Henderson (1998). These results indicate that non-JH terpenes from termite soldier castes can influence caste polyphenism in nestmates. The results presented here help identify part of the complex chemical communication system that termites utilize to maintain a balanced social environment.

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Fig. 1 Soldier head extract (SHE) dose-response. Worker termites were exposed to different soldier head equivalents (eq) or control treatments for 25 days. SHE was prepared in acetone. Soldier head extract alone was applied at 4 head equivalents. The number of head equivalents tested in combination with JH III was 0.5, 1, 2 and 4. Each treatment was replicated six times on three different colonies (7,8 and 9). The graphs for colonies 7,8 and 9 show cumulative avg. ± std. error presoldier induction through assay day 25 for each of the separate colonies. The graph at the bottom right shows cumulative avg. ± std. error presoldier induction for the combined colony responses. Letters represent significant differences at $p<0.05$.

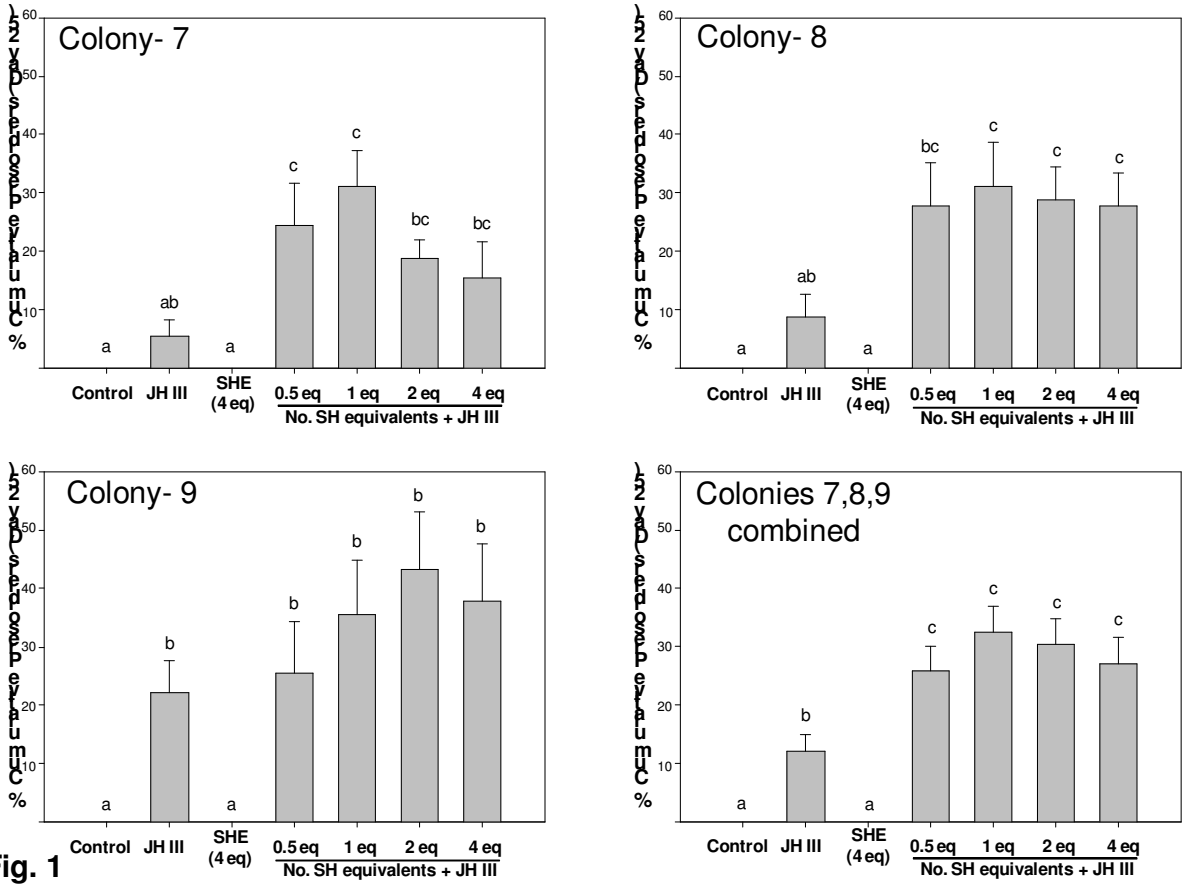

Fig. 1

Fig. 2 Analysis of soldier head, worker head, and pine extracts by gas chromatography. Gas chromatograms of acetone extracts prepared from thirty soldier (top) and worker (middle) heads, as well as 1.26 g of seasoned pine wood (bottom). Pine wood was seasoned and identical to that used to feed termite colonies.

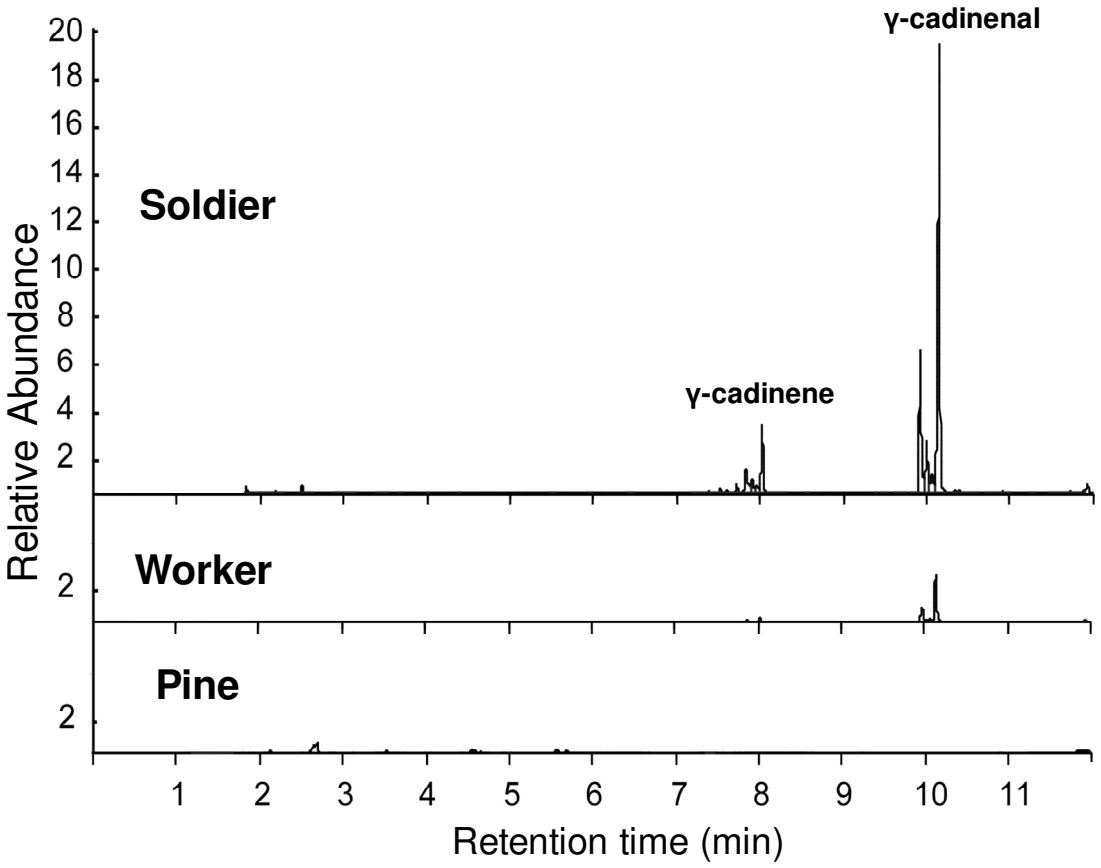

Fig. 3 Previously described soldier-derived terpenes synergistically enhance JH-dependent presoldier differentiation. Twelve previously identified soldier chemicals (mono-, sesqui- and di-terpenes) were tested for their ability to induce presoldier differentiation, alone and in combination with JH III. Treatments included; negative controls (300 µl acetone), SHE (4 soldier head equivalents), humulene, β-farnesene, cadinene, geranyl linalool, linalool, farnesol, β-pinene, limonene, nootkatone, nerolidol, α-pinene, and geranyl geraniol. All soldier head chemicals were tested at 50 µg / dish, with and without JH III (150 µg). Each treatment was replicated three times. The graph shows cumulative avg. ± std. error presoldier induction through assay day 25. Letters represent significant differences at $p<0.05$.

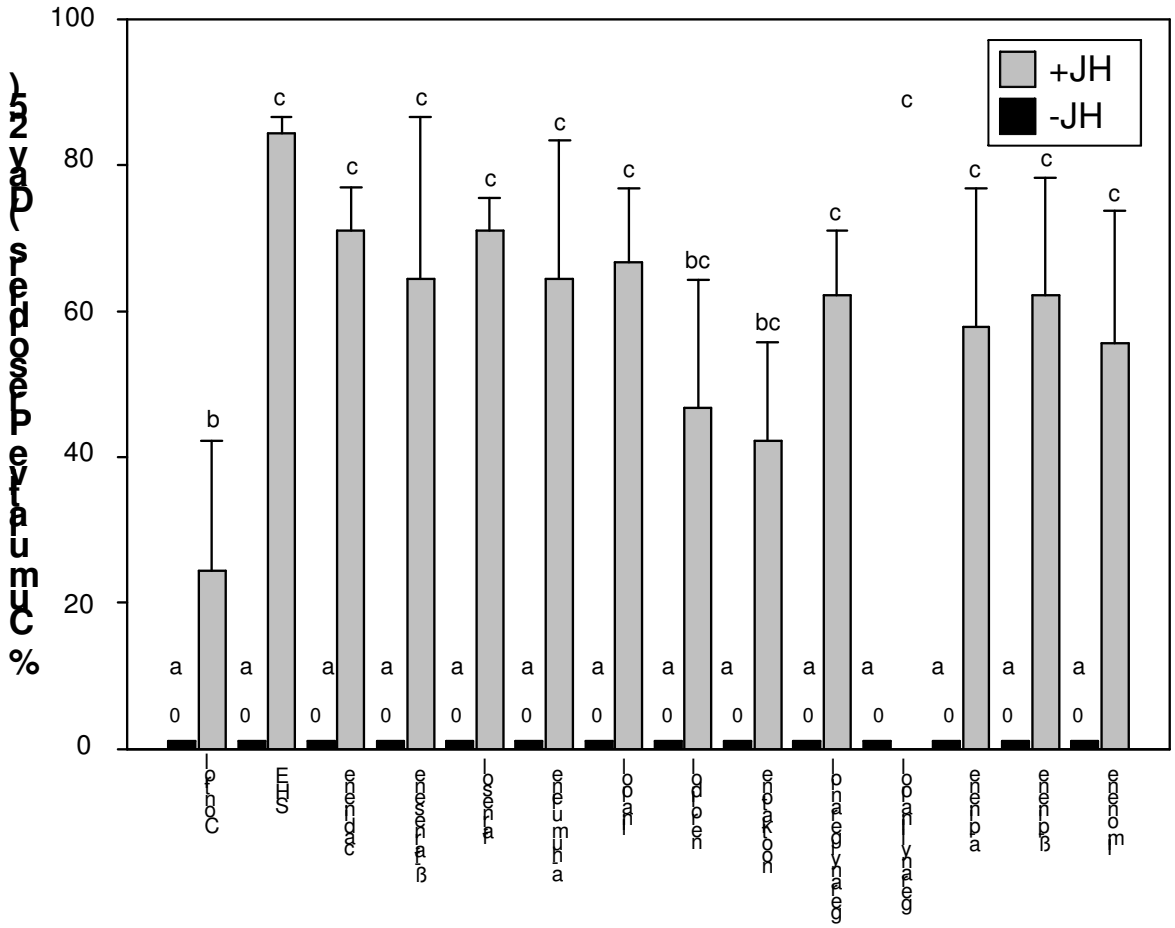

Fig. S1. Effects of soldier head extracts prepared in DCM on four *R. flavipes* colonies. Workers were isolated from colonies and exposed to five different treatments for 25 days. Soldier head extracts (SHE) were obtained by homogenizing soldier heads in methylene chloride (DCM – $MeCl_2$). The graph shows cumulative avg. ± std. error presoldier induction through assay day 25. Groups of bars with different letters indicate significant differences at $p<0.05$.

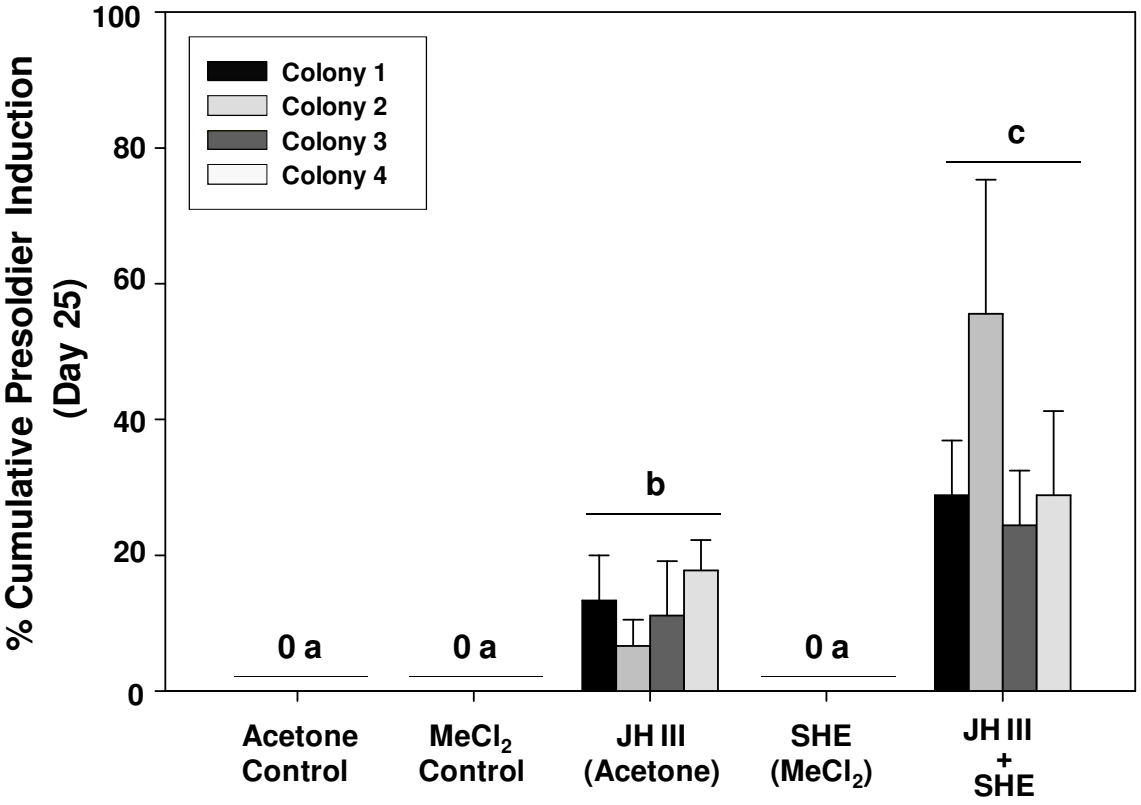

Fig. S1

Fig. S2. Analysis of soldier head extracts by mass spectrometry. Dominant compounds identified by gas chromatography were analyzed using mass spectrometry and NMR and were identified as (A) γ-cadinene and (B) γ-cadinenal. **Spectral data**: **(A)** γ-cadinene *m/z* (rel. int.): 204 (17), 189 (3), 176 (2), 161 (100), 148 (6), 133 (30), 119 (41), 105 (54), 91 (47), 79 (31), 67 (13), 55 (14), 41 (28); **(B)** γ-cadinenal *m/z* (rel. int.): 218 (19), 203 (4), 189 (4), 175 (33), 157 (49), 147 (48), 133 (61), 119 (26), 105 (72), 91 (100), 79 (63), 67 (30), 55 (27), 41 (64).