DISSERTATION

ANTIMICROBIAL AND AUTOPHOTOTOXIC EFFECTS OF NORHARMANE IN TERMITES

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MATTHEW SAMUEL SIDERHURST ENTITLED ANTIMICROBIAL AND AUTOPHOTOTOXIC EFFECTS OF NORHARMANE IN TERMITES BE ACCEPTED AS FULFILLMENT IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ANTIMICROBIAL AND AUTOPHOTOTOXIC EFFECTS OF NORHARMANE IN TERMITES

The fluorescent alkaloid norharmane has been isolated from *Reticulitermes* termites and characterized by microcoil ^{1}H NMR, UV/Vis, mass spectrometry and GC/MS. This is the first report of norharmane in insects, which is the major component in termite fluorescence under ultraviolet light. Norharmane was uniformly present at approximately 1 ng/mg in *Reticulitermes tibialis* Banks workers, soldiers and alates, *R. flavipes* (Kollar) workers and *R. virginicus* (Banks) workers. Some termites were observed to fluoresce with less intensity, but no differences in norharmane levels were detected. Norharmane occurs mainly in the termite hemolymph, and GC/MS analyses of metabolites from cultured *Reticulitermes* endosymbionts demonstrated that microorganisms are a biosynthetic origin of norharmane in termites. Norharmane shows antimicrobial activity at ecologically relevant concentrations against the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff). Subterranean termites contend with substantial microbial disease pressure due to their soil habitat and social behavior, and norharmane may be a critical defense in countering this pressure. A quantitative survey of 18 termite genera, *Anoplotermes, Amitermes, Cryptotermes, Coptotermes, Gnathamitermes, Heterotermes, Incisitermes, Kalotermes, Marginitermes, Microcerotermes, Nasutitermes, Neotermes, Paraneotermes, Prorhinotermes, Pterotermes, Reticulitermes, Tenuirostritermes* and *Zootermopsis*, from the four largest

Isopteran families, showed universal norharmane presence. Norharmane is therefore the most widespread antimicrobial defense yet reported, both across castes and phylogenetically. Norharmane was absent from five closely related taxa that were also analyzed. Norharmane is a phototoxic compound, and ultraviolet light exposure (30 and 60 W/m^2) caused high autophototoxic mortality in *Reticulitermes* termites, suggesting novel pest control possibilities. Saprophagous *Tyrophagus* sp. mites associated with laboratory colonies of *Reticulitermes* were shown to besome fluorescent after ingesting norharmane from termite cadavers, but the mites showed no mortality when exposed to UV light. No UV-mediated mortality was observed in pavement ants, *Tetramorium caespitum* L., fed on sucrose and norharmane. UV exposure bioassays with half-covered arenas showed that *Reticulitermes* termites are negatively phototactic to ultraviolet light. Autophototoxicity was significantly reduced in half-covered arenas compared to uncovered controls.

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ABBREVIATIONS

EIMS	Electron impact mass spectroscopy
ESMS	Electrospray mass spectroscopy
GC/MS	Gas chromatography/mass spectroscopy
^{1}H NMR	Proton nuclear magnetic resonance
HPLC	High performance (or pressure) liquid chromatography
HRLSIMS	High resolution liquid secondary ion mass spectrometry
LSIMS	Liquid secondary ion mass spectrometry
SIM	Selected ion monitoring
SPE	Solid phase extraction
TIC	Total ion current
TLC	Thin layer chromatography
UV/Vis	Ultraviolet/visable (spectroscopy)

CHAPTER 1

INTRODUCTION

Termites are important ecological decomposers of cellulose and with ants represent the most successful examples of insect societies (Abe et al. 2000, Hölldobler and Wilson 1990). In relation to human interests they constitute a persistent threat to homes, businesses and other structures, and are also responsible for substantial damage to forest and agricultural crops (Su and Scheffrahn 2000, Wiseman and Eggleton 1994). These social insects are among the most economically important pests with an impact that may soon reach $11 billion annually in the United States (Su 2002) and $40 billion worldwide (Wiseman and Eggleton 1994). Concurrent with increased damage from this insect is the pressure to find more environmentally friendly control methods, as several former soil termiticides have been recently deregistered (Kard 1999, Su 2002). Greater understanding of termite behavior ecology and physiology has been critical in leading to new control methods such as baiting systems and termiticides with greatly reduced mammalian toxicity (Su 2002). Gaining an understanding of the natural factors that control termite populations, such as pathogens and predators, and natural materials that have termiticidal activity, may be particularly instructive.

Serendipitous observations of fluorescence in three *Reticulitermes* species led to the identification of the fluorescent alkaloid norharmane, which is detailed in the following chapter. Norharmane has not been reported previously in insects and its biological activity in other organisms raises interesting questions about its origin and possible biological role(s) in termites.

Three previously reported sources for norharmane are of interest with regard to its occurrence in termites. 1) Norharmane has been found in a number of plant species (Allen and Holmstedt 1980, Bourke et al. 1992, Saleem et al. 2002, Tsuchiya et al. 1999)

and termites may acquire norharmane from a plant dietary source. 2) Several species of scorpions have previously been shown to contain norharmane (Stachel et al. 1999), and a similar biosynthetic pathway may be the source of norharmane in termites. 3) Norharmane is also known to be produced by two genera of Actinomycetes bacteria (Arai et al. 1976, Yomosa et al. 1987), which have representatives in the termite gut (Varma et al. 1994), and norharmane production may be the first example of an antimicrobial compound produced by a termite endosymbiont.

Norharmane is the simplest of the ß-carboline alkaloids, and shows antimicrobial activity against fungi (Quetinleclercq et al. 1995), bacteria (Oda et al. 1987, Oda et al. 1988) and toxicity effects in other organisms (Bourke et al. 1992, de Meester 1995, Yomosa et al. 1987). Termites encounter significant disease pressure (Rosengaus and Traniello 1997, Rosengaus and Traniello 2001), and the antimicrobial activity of norharmane raises the specter of its involvement in disease resistance in termites.

In addition to its 'dark' toxicity, norharmane induces potent UV-mediated phototoxicity against bacteria (Larson et al. 1988), insects (Larson et al. 1988) and eukaryotic cells (Towers and Abramowski 1983). UV levels are low in the subterranean habitats of termites (Schober and Lohmannsroben 2000), but containing norharmane may make termites susceptible to UV-mediated phototoxic effects. They may also have behavioral or biochemical mechanisms to alleviate norharmane-induced autophototoxicity.

The objectives of my dissertation were to: 1) fully characterize and quantify the presence and quantity of norharmane in *Reticulitermes* termite, 2) investigate the biosynthetic origin of norharmane in termites, 3) test the antimicrobial activity of

norharmane against an ecologically relevant pathogen, and determine the distribution of this putative defense in the Isoptera and, 4) evaluate the UV-mediated autophototoxic effects of norharmane on termites and assess resistance mechanisms to any phototoxicity.

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF NORHARMANE FROM *RETICULITERMES TIBIALIS* BANKS, *RETICULITERMES FLAVIPES* (KOLLAR) AND *RETICULITERMES VIRGINICUS* (BANKS) (ISOPTERA: RHINOTERMITIDAE) AND OBSERVATIONS ON TERMITE FLUORESCENCE

Introduction

While conducting laboratory experiments with a commercial ultraviolet lamp, we serendipitously observed fluorescence in *Reticulitermes tibialis* Banks termites from Colorado and in *R. flavipes* (Kollar) and *R. virginicus* (Banks) from Mississippi. All three *Reticulitermes* species fluoresced blue/green under ultraviolet light, with the thorax and abdomen exhibiting the most intense fluorescence on both workers and soldiers.

Fluorescence was first reported in arthropods from a number of taxa including the insects in 1954 when commercial ultraviolet lamps became readily available (Lawrence 1954, Pavan 1954a, Pavan 1954b, Pavan and Vachon 1954). Fluorescence is not a commonly reported phenomenon among arthropods but is well known in scorpions and solpugids (Cloudsley-Thompson 1978, Stahnke 1972). Limited examples of fluorescence in insects have been reported in at least 8 orders, most commonly in the Coleoptera (Stahnke 1972). However, recent work has shown fluorescence in unsclerotized larvae from a range of insect taxa that are stored products pests (Abels and Ludescher 2003). This has led to the suggestion that the possession of fluorescent chromophores may be relatively common in insect larvae with adults losing the fluorescence due to biochemical changes or cuticular sclerotization (Abels and Ludescher 2003). Ultraviolet chromophores may be common in internal fluids with fluorescence blocked by pigments such as melanin or sclerotized cuticle (Hopkins and Kramer 1992).

Whole-body fluorescence has been previously reported in the desert dampwood termite, *Paraneotermes simplicicornis* Banks, and the arid land subterranean termite, *R. tibialis* (Stahnke 1972), but the source and significance of this fluorescence has not been

investigated. Examples of fluorescent chromophores isolated from other arthropods include pterins (Ziegler and Harmsen 1969), purine uric acids (Melber and Schmidt 1992), tyrosine polymers (Messner et al. 1981), trytophan/indole derivatives (Mello and Vidal 1985, Wessing and Eichelbe 1968) and ß-carbolines (Stachel et al. 1999), but the biological roles of these compounds are poorly defined. Pterins are the most widespread fluorescent compounds, known in the Diptera (Bridges and Sohal 1980), Lepidoptera (Watt and Bowden 1966), Homoptera (Banks and Cameron 1973), Phasmida (Berthold and Henze 1971) and Orthoptera (Nemec et al. 2003).

Fluorescent methanogen bacteria have been isolated from the termite gut (Leadbetter and Breznak 1996). These bacteria are endosymbionts of gut-restricted flagellates and contain the fluorescent chromophores coenzyme F_{420} and methanopterin derivatives (Frohlich and Konig 1999, Jones et al. 1987, Leadbetter and Breznak 1996). The biological role of coenzyme F_{420} is to metabolize CO_2 and H_2 to methane (Frohlich and Konig 1999). Additionally, a second example of fluorescence in termites has been observed in hemolymph extracts from *Macrotermes natalensis* (Haviland) and is attributed to riboflavine (vitamin B_2) (Joly 1940). However, given the techniques used for this determination and the structural similarities between riboflavine and methanopterin derivatives, some uncertainty must be attached to this identification.

The objective of the present study was to isolate, characterize and quantify the fluorescent chromophore(s) responsible for whole body fluorescence in *Reticulitermes* spp. termites. In addition, observations were recorded on fluorescence in *R. tibialis, R. flavipes* and *R. virginicus* termites.

Materials and Methods

Insects. Live *R. tibialis* termites were obtained from two separate sites, Central Plains Experimental Range USDA-ARS Rangeland Resources Research Unit and a private ranch outside Nunn, Colorado. Species identification was confirmed by Dr. Boris C. Kondratieff, insect systematist at Colorado State University. The termite collection trap was a wooden frame (14 cm x 10.5 cm) filled with corrugated cardboard sheets (10 cm x 7 cm) with a wire screen on the top and bottom. Wooden fence posts around cattle and horse ranches in Colorado are frequently infested with *R. tibialis* (suggested by the late Frances Lechleitner, Colorado State University). Traps were buried in the soil 15 to 20 cm deep next to fence posts, with ~1 L of water added to moisten the surrounding soil, and this often attracted large numbers of termites within a week. Termites were then placed in plastic tubs (Rubbermaid, 4 L) containing soil (20% moisture wt/wt) and stored in the laboratory at room temperature. *Reticulitermes tibialis* alates swarms were collected in the early summer in Fort Collins, Colorado. *Reticulitermes flavipes* and *R. virginicus* were obtained from Laurel, Mississippi. Termite workers and soldiers of *Mastotermes darwiniensis* Froggatt, *Schedorhinotermes lamanianus* (Sjostedt), *Cryptotermes brevis* (Walker) and *Coptotermes acinaciformis* (Froggatt) were observed under ultraviolet light while visiting Dr. Michael Lenz at CSIRO Entomology, Canberra, Australia. Frozen *Reticulitermes* spp. (*R. tibialis* or *R. virginicus*) termites were provided by Tri-City Pest Control (Windsor, Colorado). Termites were transferred with a small paintbrush (#2, Cirrus 440 Round, Kolinsky sable, Winsor and Newton, England).

Extraction and Isolation. Frozen *R. tibialis* termites (~12 g) were ground in a tissue grinder and extracted in methanol for 6 months during laboratory renovations. The methanol extract was decanted, filtered and the solvent was removed under a nitrogen stream to give a sticky yellow residue that fluoresced under ultraviolet light. TLC showed an intense fluorescent spot at the origin and a fluorescent spot with R_f 0.48. The residue was chromatographed on silica gel (Fig. 2.1), eluting first with 5% methanol/chloroform (fractions 1-4), followed by 10% methanol/chloroform (fractions 5 and 6). Fractions were collected based on fluorescent color and intensity. Elution with additional mixtures of methanol/chloroform of increasing polarity (25 and 50% MeOH, fractions 7 and 8) failed to remove the remaining ultraviolet chromophores from the column. All fluorescent compounds were finally eluted from the column with methanol (fraction 9). Fraction 5 (Fig. 2.1), which exhibited the brightest fluorescence under ultraviolet light (blue in color), was concentrated under a nitrogen stream and purified by preparative C-18 reverse-phase HPLC. Separation was achieved with an isocratic solvent system of 60% methanol:40% 50 mM ammonium acetate buffer (pH 8) (Stachel et al. 1999). The fluorescent fractions from successive HPLC runs, retention time ~7.75 – 8 minutes, were combined and the solvent removed under a nitrogen stream to afford ~200 μg of a white solid **1,** which was subjected to spectroscopic analysis.

General Experimental Procedures. NMR spectra were recorded in d-methanol and d-chloroform on either a Varian Inova 500 spectrometer (500 MHz) equipped with a CapNMR microcoil flow probe (Magnetic Resonance Microsensors, Savoy, IL), used for mass limited samples, or a Varian Inova 300 spectrometer (300 MHz) equipped with a 5mm Quad-Nuclear plus X PFG probe, used for synthetic standards. GC/MS analysis

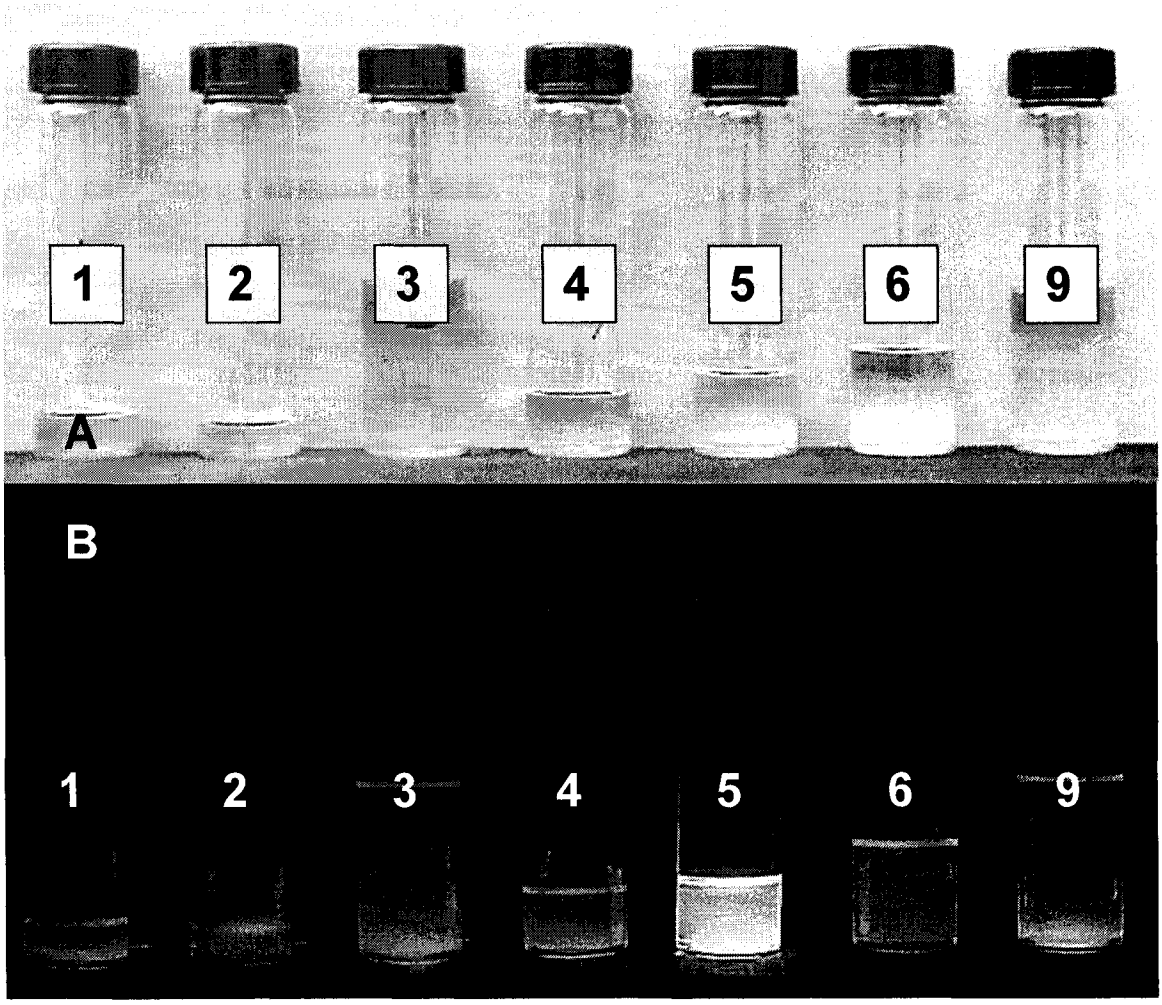


Figure 2.1 Fractions from silica gel separation of *Reticultermes* spp. methanol extract. (A) Fractions under white light. (B) Fractions under ultraviolet light. Fractions were collected based on fluorescent color and intensity. Fractions 1-4 were eluted with 5% methanol/chloroform. Fractions 5 and 6 were eluted with 10% methanol/chloroform. Fractions 7 and 8 (not shown) were eluted with 25 and 50% methanol respectively and contained no fluorescent material. Fraction 9 (methanol) eluted all remaining fluorescent compounds from the column. Fraction 5 exhibited the strongest fluorescence.

was performed on a Hewlett-Packard Series II gas chromatograph interfaced with a Hewlett-Packard 5971 Series Mass Selective Detector equipped with an Alltech Econo-Cap EC-1 column (30 m x 0.25 mm ID 0.25 μm film thickness). Two temperature programs were used, one from 120°C to 300°C at 10°C/min with a 1 min start delay or 60°C to 300°C at 10°C/min with a 1 min start delay. Low resolution and exact mass LSIMS mass spectra were recorded on a Fisons VG AutoSpec tri-sector mass spectrometer. Electron impact mass spectra were recorded on a Fisons VG Quattro-SQ single quad mass spectrometer. Electrospray mass spectra were recorded on a Finnigan LCQ-DUO ion-trap based mass spectrometer with electrospray interface. UV/Vis spectra were recorded on a Varian Cary-500 UV/VIS/NIR spectrophotometer (Plastibrand 1.5 ml semi-micro disposable cuvette, Brand GMBH + CO KG, Wertheim, Germany).

For HPLC analysis the system consisted of a Spectra-Physics SP8800 ternary pump, Valco C6W sample loop injector (10 μl loop for quantitative, 100 μl loop for preparative), and a Spectra-Physics Spectra 100 variable wavelength detector. Routine detection was at 254 nm. An Econosphere C-18 column (250 cm x 4.6 mm) (Alltech, Deerfield, Illinois) was used for both preparative separations and quantitative analysis. TLC was performed on pre-coated high performance Silica Gel 60 TLC plates (Alltech, Deerfield, Illinois). TLC plates were developed using a 75:15:10 n-butanol:water:formic acid solvent system (Stachel et al. 1999) and visualized by fluorescence under ultraviolet light. The UV source for observations of termite fluorescence was a General Electric ultraviolet lamp (model 23301, 120 VAC, 60 Hz, 16 W) equipped with a GE FL15T8-black light bulb (output 300 – 400 nm). All solvents were HPLC grade except those used for spectroscopy, which were spectroscopic grade (Sigma-Aldrich, St. Louis, MO).

Quantitative HPLC and GC/MS Analysis: Norharmane levels were analyzed in *R. tibialis* alates, soldiers and workers, along with *R. flavipes* soldiers and workers and *R. virginicus* workers. Groups of 20 termites were prepared by grinding in MeOH and then sonicated (Disontegrator System Thirty sonicator, Ultrasound Industries, Plainview, New York) for 3 hours followed by centrifugation (International Clinical Centrifuge model CL, International Equipment Co., Needham, Massachusetts). The MeOH solution was removed and filtered before being evaporated to apparent dryness with a nitrogen stream and then re-dissolved in a defined small volume (50 or 100 μl) of MeOH for HPLC and GC/MS analysis. An isocratic solvent system of 60% methanol:40% 50 mM ammonium acetate buffer (pH 8) (Stachel et al. 1999) was used for HPLC quantitative analysis. HPLC analyzes employed a standard curve for all measurements. TIC chromatographs and extracted ion chromatographs (*m/z* 168, HP Standalone Data Analysis program) were recorded in the GC/MS analysis.

Whole termite pH tests: Groups of 10 termites (*R. tibialis*, *R. flavipes* and *R. virginicus*) were ground in 250 μl of HPLC grade water. Aliquots of this aqueous solution were removed and tested with pH indicator strips (ColorpHast indicator strips, pH 0 – 14, EM Science, Gibbstown, New Jersey).

Results

Workers and soldiers of *R. tibialis*, *R. flavipes* and *R. virginicus* termites fluoresced blue/green under ultraviolet light, with the thorax and abdomen exhibiting the most intense fluorescence (Fig. 2.2). The fluorescent markings were mottled, following

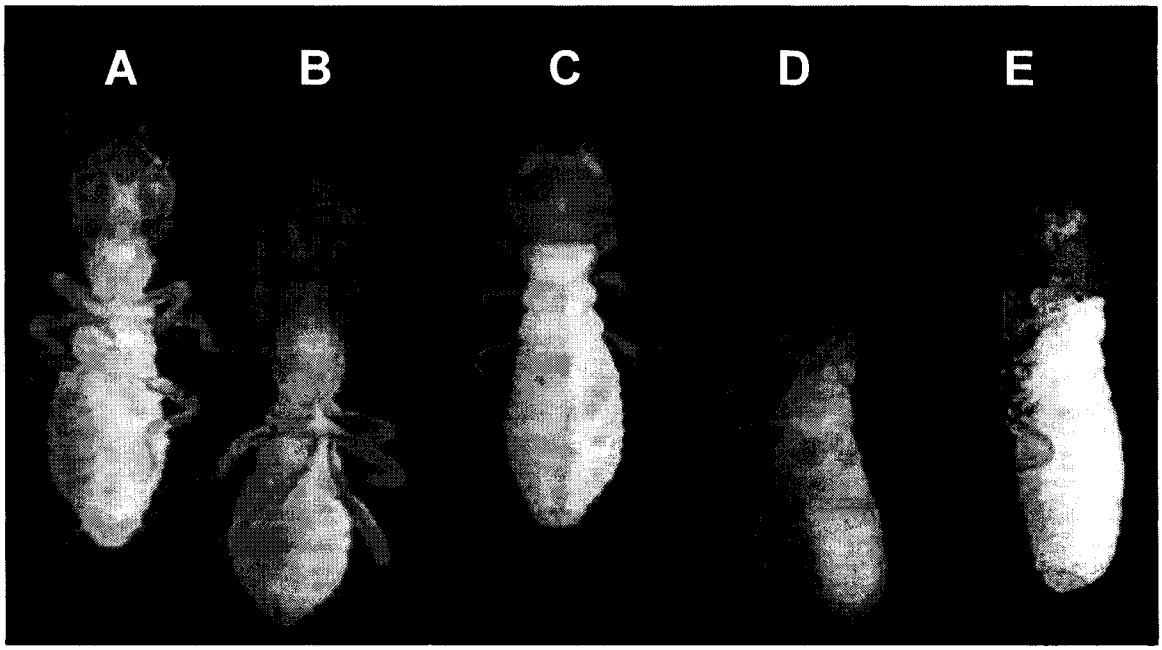


Figure 2.2 ***Reticultermes tibialis*** **termite workers and soldiers under ultraviolet light.** (A) Worker and (B) Soldier, ventral view. (C) Worker, dorsal view. (D) Soldier and (E) Worker, lateral view. Note the fluorescence surrounding the workers' eyes (C and E) and on the medial ventral surface of the head (A).

the coloration observed under white light. Additional areas of fluorescence on workers were observed surrounding the compound eyes and on the medial ventral surface of the head. Sclerotized *R. tibialis* alates did not fluoresce under ultraviolet light. Soldiers fluoresced less intensely than workers. The intensity of fluorescence varied slightly among colonies and also among individuals within colonies (Fig. 2.3). There were no obvious differences observed among individuals or groups, such as termite size or degree of sclerotization that were correlated with the fluorescent intensity variations. Of the Australian termites examined, only *Cr. brevis* fluoresced brightly under ultraviolet light with intensity patterns similar to *Reticulitermes* sp. *Mastotermes darwiniensis*, *S. lamanianus* and *Co. acinaciformis* showed only slight fluorescence, comparable to less fluorescent *Reticulitermes*. Norharmane presence and/or quantity were not analyzed in Australian spp.

Thin layer chromatography (TLC) of termite whole-body methanol extracts, visualized under ultraviolet light, showed one bright spot at R_f 0.48, a spot of moderate intensity at the origin and several additional less intense spots. The spot at the origin was presumed to be coenzyme F_{420}, which is highly polar. No attempt with made to identify the moderately fluorescent spots, which could include riboflavine and other UV chromophores already known in insects (Joly 1940, Ziegler and Harmsen 1969). Silica gel separation yielded a single fraction that showed strong ultraviolet fluorescence. Purification by preparative HPLC followed afforded relatively pure material **1** for spectroscopic analysis.

UV/Vis spectrum for **1** had absorbances at 235, 290 and 350 nm, which is characteristic of the β-carboline moiety (Fig. 2.4) (Parameswaran et al. 1997). $ESMS^+$ of

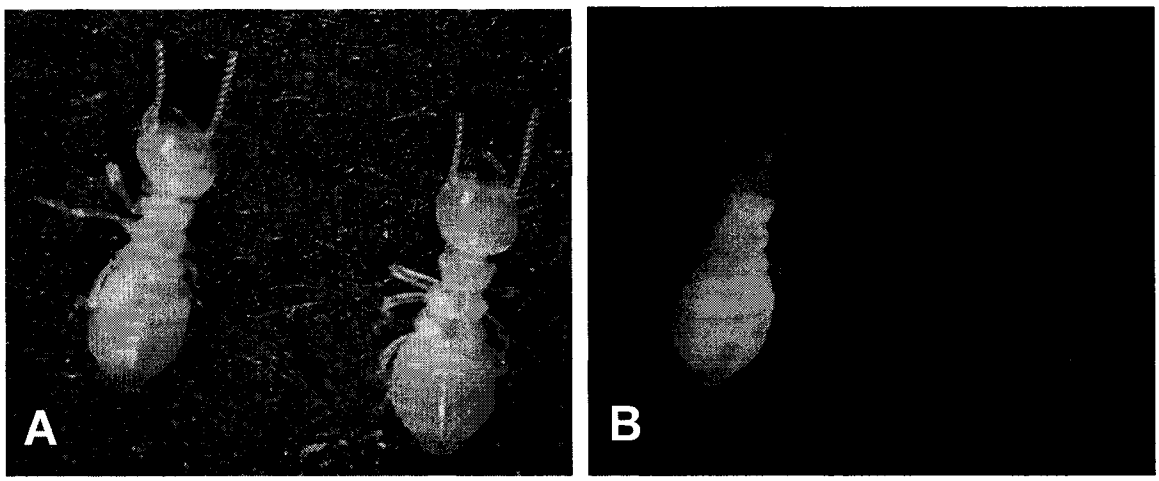
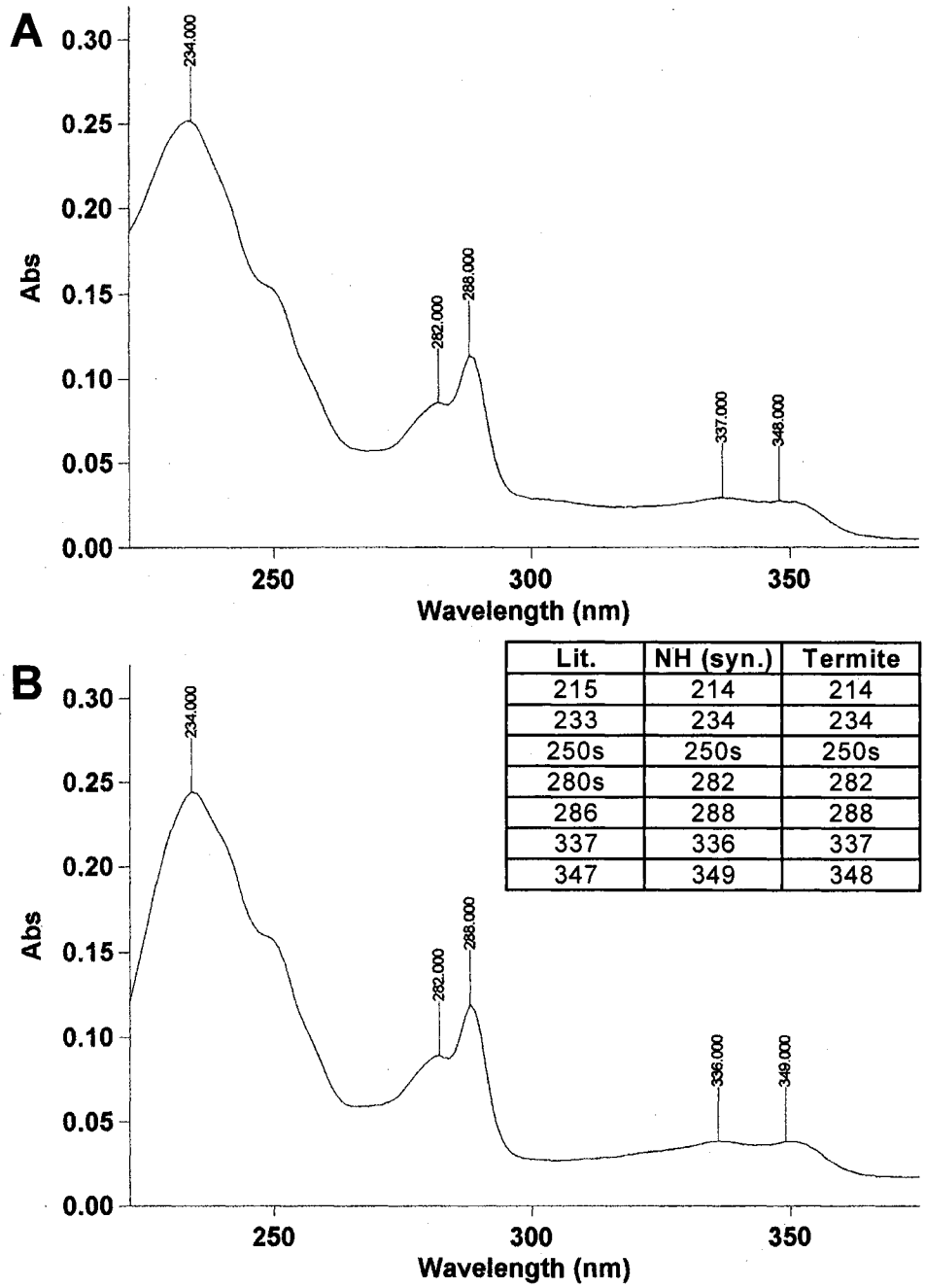


Figure 2.3 Fluorescent variation in *Reticultermes tibialis.* Natural variations are shown between two members of the same colony, under white (A) and ultraviolet light (B). HPLC analyses showed both termites have ~1 ng of norharmane per termite. Intensity variations may be due to pH differences at the tissue level or may be due to quenching compounds.



Lit.	NH (syn.)	Termite
215	214	214
233	234	234
250s	250s	250s
280s	282	282
286	288	288
337	336	337
347	349	348

Figure 2.4 UV/Vis spectra. (A) Purified norharmane from *Reticulitermes* spp. (**1**). (B) Synthetic norharmane. Literature values from Balon et al. (1993).

1 displayed the molecular ion peak at *m/z* 169.07 with high resolution LSIMS$^+$ provided the molecular formula $C_{11}H_9N_2$ for the protonated species. EIMS showed peaks at *m/z* 168, 140 and 114. ^{1}H NMR spectroscopy in d-chloroform proved unhelpful due to overlap of the solvent peak and spinning sidebands and peaks of interest (Fig. 2.5). Initial ^{1}H NMR spectra in d-metanol displayed the characteristic spectrum for norharmane shifted downfield by ~0.1 - 0.2 ppm. Addition of synthetic norharmane to **1** (1:1 and 2:1, synthetic:**1**) showed that all peaks of interest coalesced at the downfield ppm values (Fig. 2.5). Downfield shifts of ~0.1 ppm were observed with synthetic norharmane upon addition of 1% deuterated glacial acetic acid. TLC, GC/MS and HPLC chromatographic data were consistent between **1** and norharmane (Fig. 2.6). Methanol extracts from all three *Reticulitermes* spp. were analyzed by TLC with the fluorescent spot at R_f 0.48 matching synthetic norharmane (Fig. 2.7).

Norharmane (9H-pyrido[3,4-b]indole) (**1**): white amorphous solid; UV/Vis (MeOH) λ_{max} 214, 234, 252, 282, 288, 337, 348 nm (synth. 214, 234, 252, 282, 288, 336, 349 nm); ^{1}H NMR (CD_3OD, 500 MHz) δ 8.927 (1H, s, H-1), 8.353 (1H, d, H-3), 8.302 (1H, d, H-1), 8.286 (1H, d, H-4), 7.658 (2H, m, H-5/7), 7.356 (1H, m, H-6), (synth., CD_3OD, 300 MHz) δ 8.797 (1H, s, H-1), 8.293 (1H, d, *J* = 5.4 Hz, H-3), 8.200 (1H, d, *J* = 7.9 Hz, H-1), 8.094 (1H, d, *J* = 5.4 Hz, H-4), 7.577 (2H, m, H-5/7), 7.282 (1H, m, H-6); ESMS$^+$ *m/z* 169.07 $[M + H]^+$; FAB$^+$ *m/z* 169.07 $[M + H]^+$; HRLSIMS$^+$ *m/z* 169.0765 $[M + H]^+$ (calcd for $C_{11}H_9N_2$, 169.07657); EIMS *m/z* 169 $[M + 1]^+$ (14), 168 $[M]^+$ (100), 167 $[M - 1]^+$ (14), 140 (35), 114 (26), 84 (44), 70 (43); GC/MS ret. time 17.10 min. (synth. 17.07 min.), *m/z* 169 $[M + 1]^+$ (13), 168 $[M]^+$ (100), 167 $[M - 1]^+$ (9), 140 (16), 114 (7), 84 (5), 70 (3).

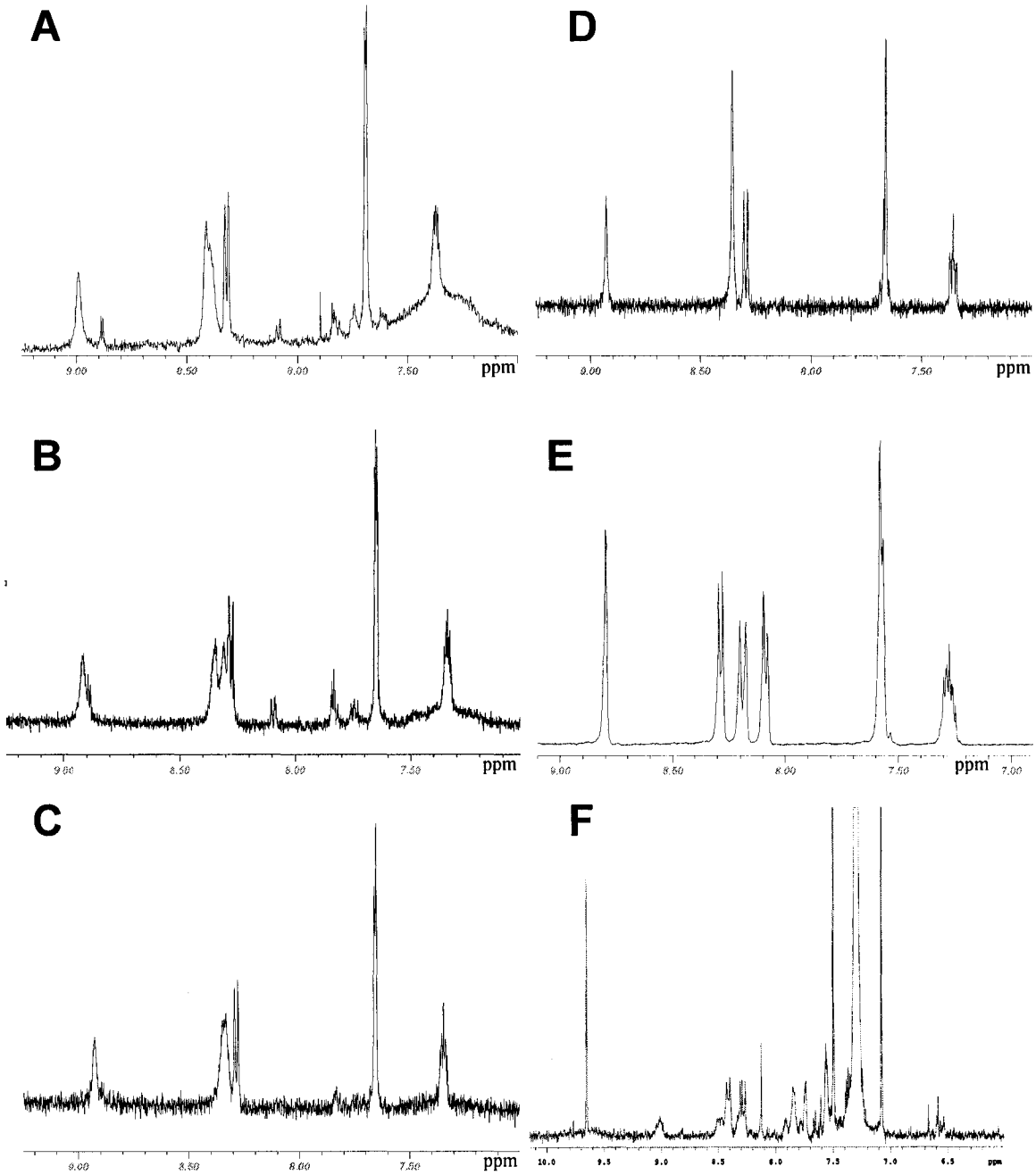


Figure 2.5 ^{1}H NMR spectra. (A & F) Purified norharmane from *Reticulitermes* spp. (**1**). (B) Synthetic norharmane and **1** (1:1). (C) Synthetic norharmane and **1** (2:1). (D) Synthetic norharmane and 1% deuterated glacial acetic acid. (E) Synthetic norharmane. (A-D) Microcoil probe, 500 MHz ^{1}H NMR in CD_3OD. (E) Standard probe, 300 MHz ^{1}H NMR in CD_3OD. (F) Standard probe, 500 MHz ^{1}H NMR in $CDCl_3$.

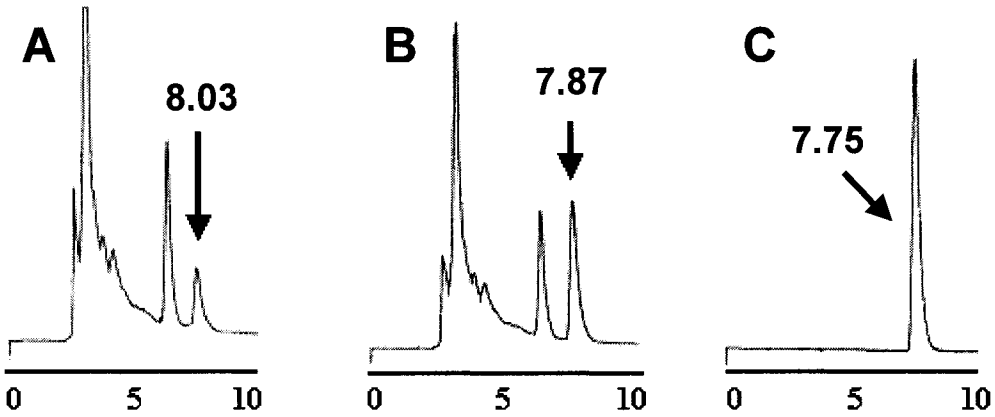


Figure 2.6 HPLC analyzes. (A) Purified norharmane from *Reticulitermes* spp. (**1**). (B) Synthetic norharmane and **1** (co-chromatographing). (C) Synthetic norharmane. The separation was achieved using an isocratic solvent system of 60% methanol:40% 50 mM ammonium acetate buffer (pH 8).

Figure 2.7 TLC of norharmane from arthropods. (A-C) *Reticultermes tibialis,* (D-F) synthetic norharmane (decreasing amounts), (G) *Reticultermes flavipes* and (H) *Reticultermes virginicus*. Silica gel TLC plates were developed with 75:15:10 n-butanol:water:formic acid and visualized by fluorescence under ultraviolet light.

HPLC quantitative analysis showed *R. tibialis, R. flavipes* and *R. virginicus* workers contain approximately 1 ng of norharmane per termite, and *R. tibialis* and *R. flavipes* soldiers contained slightly higher amounts (Table 2.1). Surprisingly, HPLC analysis showed no differences in norharmane levels between groups of termites that fluoresced brightly and those that fluoresced weakly. Whole termites were ground in water and the pH was tested, because pH is known to affect the fluorescent properties of ß-carboline (Balon et al. 1993). No pH differences were detected in the water extracts, which might have explained the varying fluorescence.

Discussion

Reticulitermes termites encounter little or no ultraviolet light in their natural habitats (Schober and Lohmannsroben 2000), and it is possible that the fluorescence of norharmane is incidental to its biological role. However, the fluorescence surrounding the eyes of *Reticulitermes* workers shows that these areas are opaque to ultraviolet light and may allowing exposure of UV-photosensors in the head. UV chromophores are known to be involved in insect sight (Eckert 1971, Kay 1969) and photoreceptors have been reported from within the brain (protocerebrum) itself (Fleissner and Fleissner 2003, Gao et al. 1999). There is also precedence for photoreception that involves areas of modified cuticle on the head that allow penetration of restricted frequencies of light (Hardie et al. 1981, Meyer 1977, Seifert et al. 1987).

Compound **1**, the predominant fluorescent chromophore in *Reticulitermes* spp. termites, was identified as norharmane by comparison of spectral data from capillary ^{1}H

Table 2.1 Norharmane levels in *Reticulitermes* spp. termites. Concentrations of norharmane in *Reticulitermes* species and castes were determined by single replicate analyzes of 20 termites batches by HPLC or GC/MS. (A) Termites with strong fluorescent intensity, (B) termites with weak fluorescent intensity.

	Workers				Soldiers		Alates
	R. tibialis **A**	*R. tibialis* **B**	*R. flavipes*	*R. virginicus*	*R. tibialis*	*R. flavipes*	*R. tibialis*
ng/termite (HPLC)	1.1	1.2	1.3	1.1	--	--	3.2
ng/termite (GC/MS)	1.2	--	--	--	2.9	7.9	--
ng/mg (HPLC)	0.6	0.6	0.7	0.6	--	--	0.6
ng/mg (GC/MS)	0.6	--	--	--	1.0	1.6	--

NMR, UV/Vis, HRLSIMS, electrospray, EIMS and GC/MS with synthetic norharmane and literature values (Balon et al. 1993, Parameswaran et al. 1997, Stachel et al. 1999). This was further confirmed by TLC and HPLC chromatographic data. Microcoil ^{1}H NMR spectroscopy allowed spectra to be obtained from mass-limited material facilitating the identification of this biologically scarce natural product.

Attempts to identify natural products in biological systems are often hampered by the minute amounts of material that can be obtained from a given organism, particularly organisms that are small and relatively scarce. NMR spectroscopy is one of the most powerful techniques available for total structural identification, but its relatively low sensitivity often precludes its use with mass-limited natural products (Putzig et al. 1994). NMR sensitivity is proportionally related to increasing magnetic field strength and considerable effort has been expended in producing higher field instruments. The development of highfield magnets that are stable, maintain high quantity field homogeneity and are economical, has so far proved an elusive goal (Wolters et al. 2002). Two recent advances in NMR probe design have begun to address this problem by improving signal-to-noise ratios without moving to higher field strength instruments. Cryogenic probes supercool the detection electronics thereby cutting down background noise, which increases sensitivity (Black et al. 1993, Flynn et al. 2000). Microcoil probes increase the signal-to-noise ratio by reducing the diameter of the NMR coil, which is inversely related to mass-sensitivity (Olson et al. 1995, Wolters et al. 2002). Although cryogenic probes attain greater molar sensitivity, microcoil probes are currently less expensive and can better handle samples that have lower absolute masses.

Norharmane has marked changes in its absorbance and emission spectra in a pH dependent fashion. This is the result of four acid-base dependent species, neutral, cationic, zwitterionic and anionic (Fig. 2.8) (Balon et al. 1993). In addition, a fifth species (P) is proposed in which groups of norharmane molecules interact (Reyman et al. 1997). Given this pH selectivity, the downfield shifting of peaks in the ^{1}H NMR spectra of **1,** and synthetic norharmane with 1% deuterated glacial acetic acid, are consistent with protonation of the nitrogen in the pyridine ring (N-2). The resulting cation molecular species experiences greater deshielding due to the stronger electron withdrawing effect from the pyridine ring nitrogen. Downfield shifts are most pronounced for H-1 and H-3, which is consistent with the greatest deshielding effects occurring adjacent to N-2.

This is the first report of norharmane in insects, but it has been previously isolated from several species of scorpions, perhaps the best-known examples of fluorescence in arthropods (Stachel et al. 1999). Norharmane in scorpions is thought to be a by-product of cuticular sclerotization, but there is no proposed ecological significance for its presence (Stachel et al. 1999). Apart from norharmane, the only previous record of a ß-carboline in insects is 1,2,3,4,-tetrahydro-beta-carboline-3-carboxylic acid (THCA), which occurs in larvae of the yellow mealworm, *Tenebrio molitor* L. (Kotanen et al. 2003). THCA is a defensive compound and belongs to a group of molecules termed "paralsins" for their highly toxic effects on adult insects of the same species and other species (Kotanen et al. 2003).

Norharmane is the simplest of the ß-carboline alkaloids, which have a wide distribution in the plant kingdom (Allen and Holmstedt 1980) and occur with lower

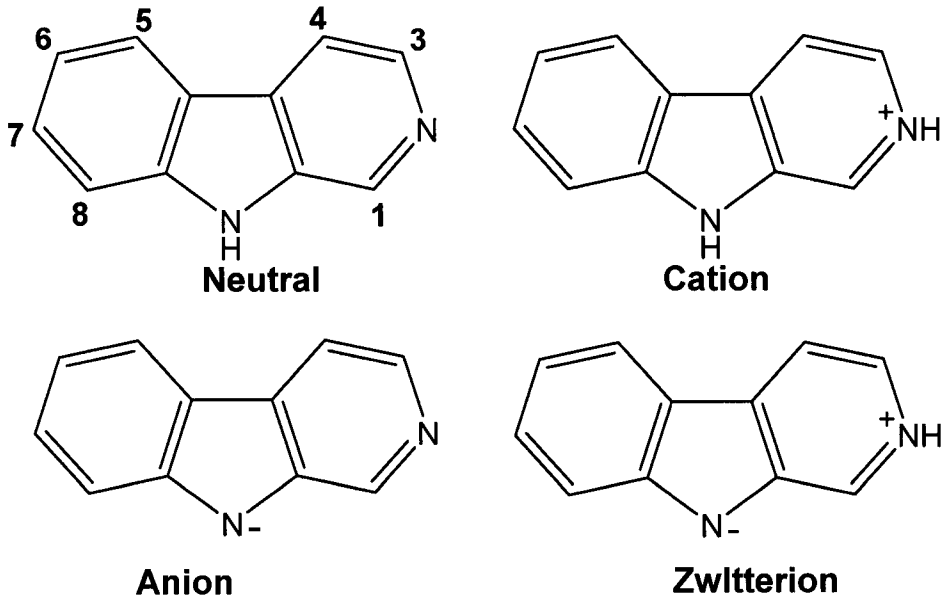


Figure 2.8 Chemical structures of norharmane species.

frequency in other taxa. The distribution of ß-carbolines is thought to reflect their broad spectrum of toxicity and phototoxicity against a variety of organisms (Allen and Holmstedt 1980). Norharmane has shown toxicity against fungi (Quetinleclercq et al. 1995) and plant seedlings (Yomosa et al. 1987), causes SOS responses and frame shift mutations in bacteria (Oda et al. 1987, Oda et al. 1988), sublethal nervous effects in mammals (Bourke et al. 1992), and has broad mutagenic/co-mutagenic properties (de Meester 1995). Toxic effects of norharmane that are of most relevance to insects may be its inhibition of cytochrome P_{450} (Nii 2003), which are a major class of detoxification enzymes in insects (Feyereisen 1999). DNA intercalation of norharmane (Nii 2003) and oxidative DNA damage caused by aminophenylnorharmane formed from norharmane and aniline may be mechanisms of toxicity (Masumura et al. 2003, Ohe et al. 2002, Ohnishi et al. 2001, Totsuka et al. 2002). Closely related simple β-carbolines have shown some toxicity toward GABA receptors in the insect nervous system (Bloomquist et al. 1997, Hosie and Sattelle 1996).

Norharmane also exhibits potent phototoxic activity against bacteria (Larson et al. 1988), insects (Larson et al. 1988) and eukaryotic cells (Towers and Abramowski 1983). Other ß-carbolines also exhibit phototoxicity towards viruses (Hudson et al. 1986) and fungi (McKenna and Towers 1981). In light of its toxic activity, norharmane is well suited to serve as a chemical defense in termites.

The intensity of fluorescence in all three *Reticulitermes* species was greatest in the abdomen and thorax, the regions of the body with least sclerotization. Of the Australian spp. examined, only the least sclerotized, *Cr. brevis*, showed fluorescence comparable to *Reticulitermes* spp., although no norharmane analyses were conducted on *Cr. brevis*, *M.*

darwiniensis, *S. lamanianus* or *Co. acinaciformis*. HPLC analysis of *Reticulitermes* spp. showed that all species and castes tested had approximately the same concentrations of norharmane. No differences were found in norharmane levels among groups of *Reticulitermes* termites that fluoresced brightly and those that fluoresced weakly. Similarly, *R. tibialis* and *R. flavipes* soldiers were observed to have less intense fluorescence, but they had levels of norharmane comparable to those of workers of the same species. This suggests that termites may have mechanisms that regulate their fluorescent intensity without changing the concentration of the fluorescent chromophore, which in the case of soldiers may be a caste-wide phenomenon.

Acidity affects norharmane fluorescence strongly, with the cation displaying higher fluorescent intensity than the neutral species (Balon et al. 1993). Although no differences in pH were found when testing whole termites, pH differences at the tissue level may affect fluorescent intensity. Admittedly, the techniques used in this study for pH detection were crude, and employing techniques with greater sensitivity to pH within insect tissue (Harrison 2001), may reveal differences that account for the observed fluorescence variation in *Reticulitermes* termites. Insects can regulate the pH of their internal fluids such as hemolymph and gut contents (Harrison 2001). In particular pH changes are known to assist in dealing with toxins (Govenor et al. 1997, Timmermann et al. 1999). Acetic and lactic acids are both essential metabolites that termites (or their endosymbionts) derive from the digestion of cellulose (Breznak 2000, Inoue et al. 2000). Systemic alkalinity due to nutritional deficiencies could cause the decreased fluorescence seen in some termites.

Partitioning experiments with aqueous/organic microemulsions and norharmane have shown a marked effect on fluorescence by altering the equilibrium between the neutral and cationic species (Varela et al. 1995). In pure non-polar solvents only the neutral species is observed, while in pure polar solvents, pH largely determines the molecular species equilibrium, with norharmane solubility increasing at high and low pH. Mixed solvent systems, which more closely resemble biological systems, show an interaction among pH, solvent polarity and surface interactions that determine the species equilibrium. Termite fluorescence may decrease as a function of increasing lipid concentration in the body fluids as this favors the neutral species over the cation.

Another possibility is that these termites may quench norharmane fluorescence by enzymatic means, such as superoxide dismutase, catalase or glutathione reductase (Larson 1986, Pritsos et al. 1988). Alternatively, they may contain compounds that absorb ultraviolet light and thereby prevent norharmane from fluorescing such as ascorbic acid, carotenes (Berenbaum 1987) or tocopherols (Felton and Summers 1995). This may be particularly important in the fluorescent variations observed between worker and soldier, as these castes have different cuticular permeabilities (Shelton and Grace 2003). If quenching compounds are present, they may reduce any phototoxic risks associated with containing the phototoxin norharmane, especially for termites that forage (workers) or perform defensive duties (soldiers) outside the nest.

Termites constitute a persistent threat to homes, businesses and other structures. These social insects are among the most economically important pest with an impact that may soon reach $11 billion annually in the United States (Su 2002) and $40 billion worldwide (Wiseman and Eggleton 1994). Inspecting structures for termite presence and

damage constitute an important part of minimizing the effects of this pest. In his initial observation of fluorescence in *P. simplicicornis* and *R. tibialis,* Stahnke (1972) noted that ultraviolet light made detection of even single termites much easier then detection in white light. Ultraviolet light induced fluorescence as a means of detecting insects has been proposed for a number of larval stored products pests (Abels and Ludescher 2003) and this technique may be helpful in the detection of termites as well.

Although norharmane is a normal body constituent (Fekkes et al. 1992), it has been implicated in several human health disorders, most notably addiction and Parkinson's disease. Norharmane involvement in addiction has been studied in laboratory animals and/or clinical trial in relation to morphine (Aricioglu-Kartal et al. 2003, Cappendijk et al. 1994a, Cappendijk et al. 1994b), cocaine (Cappendijk et al. 2001), heroin (Stohler et al. 1995) and alcohol and tobacco (Badawy et al. 1998, BreyerPfaff et al. 1996, Fekkes et al. 2002, Kiefer et al. 2000, Rommelspacher et al. 1991, Rommelspacher et al. 1996, Spies et al. 1995, Spies et al. 1996, Spijkerman et al. 2002). Elevated levels of norharmane and other β-carbolines have been found in Parkinson's disease patients (Kuhn et al. 1995b, Kuhn et al. 1995a, Kuhn et al. 1996) and there is some evidence that norharmane may disrupt nervous system function in the brain (Gearhart et al. 2000, Matsubara et al. 1998). Norharmane has also been linked to mental retardation (Tuinier et al. 2000, Verhoeven et al. 1999) and anxiety disorders (Pepplinkhuizen et al. 1996, Verheij et al. 1997), while closely related β-carbolines are linked to "essential tremor" (Louis et al. 2002). Insects and particularly *Drosophila melanogaster* are becoming increasingly important as models for research on human neurological diseases (Schneider 2000, Tickoo and Russell 2002). An example of this is

seen with the use of the '*Drosophila* model' to investigate the physiological roles of compounds involved in Parkinson's disease (Bonini and Fortini 2003, Dawson and Dawson 2003, Feany and Bender 2000, Greene et al. 2003, Scherzer et al. 2003, Shulman et al. 2003). Termites may offer a similar model system to investigate these disorders and provide insight into physiological and biochemical mechanisms for coping with relatively high norharmane levels.

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CHAPTER 3

ENDOSYMBIONT BIOSYNTHESIS OF NORHARMANE IN *RETICULITERMES* TERMITES (ISOPTERA: RHINOTERMITIDAE)

Introduction

Norharmane has been identified as the predominant fluorescent chromophore in *Reticulitermes* termites and is known from 18 termite genera distributed among 4 families (Siderhurst 2004a, Siderhurst 2004b). Norharmane shows antimicrobial activity against the entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) and may be a critical defense in countering the significant disease pressure experienced in the subterranean habitat of termites (Siderhurst 2004a). Norharmane is also a phototoxin, and ultraviolet light exposure caused autophototoxic mortality in *Reticulitermes* termites, suggesting novel pest control possibilities (Siderhurst 2004c). *Reticulitermes* termites are negatively phototactic to ultraviolet light, which may help to minimize autophototoxicity. The biosynthetic origin of norharmane in termites has not yet been investigated but three possible sources are apparent: 1. they may acquire norharmane from a dietary source, 2. norharmane may be a by-product of cuticular sclerotization as speculated in scorpions (Stachel et al. 1999), or 3. endosymbiotic bacteria or protists may be the biosynthetic origin of the alkaloid.

It does not seem likely that termites feed heavily on plant species that contain ß-carbolines, so it is doubtful that they acquire norharmane directly from their dietary plant sources. Three major nutritional strategies are known in termites, soil-feeding (Bignell 1994, Brauman et al. 2000), fungus culturing (Aanen et al. 2002, Abe et al. 2000, Batra and Batra 1979) and wood-feeding (Abe et al. 2000). *Reticulitermes* termites are mainly wood feeders, as are many species of lower termites (Wood 1978). They forage for and consume cellulosic material (often dead wood) away from the net site. On returning to

the colony, nestmates that do not forage, such as soldiers and alates, are fed by trophallaxis (Traniello and Leuthold 2000). Preferences for particular woods and increased fitness while feeding on these woods have been shown with the Formosan subterranean termite, *Coptotermus formosanus* Shiraki (Morales-Ramos and Rojas 2001, Morales-Ramos and Rojas 2003a, Morales-Ramos and Rojas 2003b). These preferred woods are generally faster growing trees with soft wood such as birch, sugar maple and aspen. Norharmane has been found in a number of plant species (Allen and Holmstedt 1980, Bourke et al. 1992, Saleem et al. 2002, Tsuchiya et al. 1999). However, these plants are often shrubs with limited geographic distribution and density in ecosystems, and do not fit the general food source characteristics of termites.

Norharmane has previously been reported in several species of scorpion (Stachel et al. 1999) and a similar biosynthetic pathway may be the source of norharmane in termites. In scorpions, norharmane is confined to the thin epicuticle layer of their exoskeleton (Pavan 1954a, Pavan 1954b) where it is proposed to be a by-product of cuticular sclerotization (Stachel et al. 1999). This oxidative process of protein cross-linking is thought to facilitate the formation of norharmane from tryptophan, in part by a Pictet-Spengler reaction. Norharmane synthesis as a by-product of cuticular sclerotization is proposed to be analogous to cataract formation in human eyes although the exact mechanism is still unknown (Stachel et al. 1999). *Reticulitermes* termites are not as heavily sclerotized as scorpions, but extensive sclerotization may not be required to produce the levels of norharmane found in termites.

The possibility that endosymbionts might be the biosynthetic source of norharmane in termites was suggested by the production of norharmane by

Actinomycetes bacteria (Arai et al. 1976, Yomosa et al. 1987), which have representatives in the termite gut (Varma et al. 1994). Termites owe much of their ecological success to symbiotic bacteria, fungi and protists. Endosymbionts are nutritionally important to termites because they digest cellulose, affect nitrogen fixation and produce acetate from CO_2 (Abe et al. 2000). Two genera of soil bacteria, *Nocardia* sp. and *Streptomyces lavendulae* (Waksman and Curtis), which both belong to the Actinomycetes, are known to produce norharmane and its methylated analog harmane (Arai et al. 1976, Yomosa et al. 1987). Actinomycetes, and *Streptomyces* spp. in particular, are known components of the termite gut microfauna (Schafer et al. 1996, Varma et al. 1994), suggesting that norharmane may have its biosynthetic origin in an endosymbiont. This would not be without precedence as the β-carboline harmane is known to be produced by a tunicate-associated bacterium (Aassila et al. 2003).

The objective of the present study was to elucidate the biosynthetic origin of norharmane in *Reticulitermes* termites through feeding bioassays, histological examination and isolation and extraction of endosymbionts and their metabolites.

Materials and Methods

Insects. *Reticulitermes tibialis* Banks termites were obtained from two separate sites, Central Plains Experimental Range USDA-ARS Rangeland Resources Research Unit and a private ranch outside Nunn, Colorado. Species identification was confirmed by Dr. Boris C. Kondratieff, insect systematist at Colorado State University. The termite collection trap was a wooden frame (14 cm x 10.5 cm) filled with corrugated cardboard

sheets (10 cm x 7 cm) with a wire screen on the top and bottom. Wooden fence posts around cattle and horse ranches in Colorado are frequently infested with *R. tibialis* (suggested by the late Frances Lechleitner, Colorado State University). Traps were buried in the soil 15 to 20 cm deep next to fence posts, with ~1 L of water added to moisten the surrounding soil, and this often attracted large numbers of termites within a week. Termites were then placed in plastic tubs (Rubbermaid, 4 L) containing soil (20% moisture, wt/wt) and stored in the laboratory at room temperature. *R. flavipes* (Kollar) were obtained from Laurel, Mississippi. Termites were transferred with a small paintbrush (#2, Cirrus 440 Round, Kolinsky sable, Winsor and Newton, England).

General Experimental Procedures. GC/MS analysis was performed on a Hewlett-Packard Series II gas chromatograph interfaced with a Hewlett-Packard 5971 Series Mass Selective Detector equipped with an Alltech Econo-Cap EC-1 column (30 m x 0.25 mm ID 0.25 μm film thickness). The temperature program used ramped from 120°C to 300°C at 10°C/min with a 1 min start delay. TLC was performed on pre-coated high performance silica gel 60 TLC plates (Alltech, Deerfield, Illinois). TLC plates were developed using a 75:15:10 n-butanol:water:formic acid solvent system (Stachel et al. 1999) and visualized by fluorescence under UV light. A Disontegrator System Thirty sonicator (Ultrasound Industries, Plainview, New York) was used for sonication. Samples were centrifuged was a International Clinical Centrifuge model CL (International Equipment Co., Needham, Massachusetts). Observations and photography were performed with a Leica/Wild M3Z stereozoom microscope (E. Licht Co., Denver, CO). Digital images were recorded with a Nikon 960i digital camera (Nikon USA, Melville, NY). The UV source for observations of termite and endosymbiont

fluorescence was a General Electric UV lamp (model 23301, 120 VAC, 60 Hz, 16 W) equipped with a GE FL15T8-black light bulb (output 300 – 400 nm). All solvents were HPLC grade (Sigma-Aldrich, St. Louis, MO). All reagents used were purchased from Sigma-Aldrich, St. Louis, MO.

Histological Examination: *Reticultermes tibialis* and *R. flavipes* were frozen and placed on a cold dissection plate (a small styrofoam box, 12 cm x 16 cm x 16 cm, filled with dry ice and topped with a glass plate). Termites were sectioned with a scalpel and the sections were transferred immediately to chilled slides for examination under a dissecting scope illuminated with UV light.

Hemolymph Removal: Hemolymph was removed from *R. tibialis* and *R. flavipes* termites for analysis. The termites were frozen and then allowed to stand at room temperature in Pyrex petri dishes lined with Anchor Steel Blue Seed Germination Blotter paper moistened with distilled water. Under these conditions the cuticles of some termites ruptured and beads of hemolymph formed on the termites. Other termites were punctured with a sharp needle to release hemolymph. The fluorescent hemolymph was collected in 10 µl microcapillary pipettes (Drummond Scientific Co., Broomall, PA), which were sealed with Parafilm M (American National Can, Menasha, WI) until analyses were performed by TLC and GC/MS.

Isolation and Culturing of Endosymbionts: Groups of 10 termite workers (*R. tibialis* or *R. flavipes*) were surface-sterilized in 95% ethanol to kill any microbes on the termite cuticle. The termites were allowed to dry and then ground in sterile distilled water. Aliquots (1 ml) of the aqueous termite extract were inoculated onto plates of Sabouraud dextrose agar with 1% yeast extract (mycological peptone, 10g/L; dextrose,

40g/L; agar, 15 g/L; yeast extract, 1 g/L) (Reeves 2002) and spread with a flamed loop to give a 10^4 dilution series. The plates were incubated at room temperature for 5 days and then examined under UV light. Colonies that showed strong fluorescence were transferred to new plates.

Extraction, Isolation and GC/MS Analysis: Media from plates containing fluorescent material were extracted by sonication in MeOH for 3 hours followed by removal of the methanolic solution. The solution was centrifuged to remove visible tissue, filtered, and the volume was reduced with a nitrogen stream. The residue was adsorbed onto pre-packed High Flow C-18 Extract-Clean solid phase extraction (SPE) columns (Alltech, Deerfield, Illinois) and eluted sequentially with water, water/MeOH and MeOH. The MeOH fractions were evaporated to apparent dryness with a nitrogen stream and then re-dissolved in a small volume (10-100 μl) of MeOH for GC/MS analysis. Samples of the crude residue before C-18 purification were also analyzed by GC/MS. TIC chromatographs and extracted ion chromatographs (*m/z* 168, HP standalone data analysis program) were obtained from GC/MS analysis.

Termite Feeding Bioassay: Groups of approximately 100 termites (*R. tibialis,* workers and soldiers) were placed in a plastic tub (Rubbermaid, 414 ml) that contained soil (20% moisture, wt/wt), and were allowed to acclimate for 24 hours. Whatman #4 filter paper (Whatman International Ltd., Maidstone, England) was cut into 4 cm x 4 cm squares. Individual squares of filter paper were placed on a platform made by cutting the bottom from a plastic cup (3 cm diam.). This was turned upside down to provide a platform to pin (Singer ball point steel pins, size 17) the 4 cm x 4 cm squares of filter paper. A pencil line was drawn down the center of this square denoting the two treatment

sides. Solutions of norharmane in methanol (2.5 or 25 μg/μl) or a methanol control were applied to the filter paper with a Hamilton syringe (100 μl). A single filter paper square weighed 9.5 mg/cm^2, and this was evenly wetted with 30 μl of the methanol solutions resulting in test concentrations of 0%, 0.1% and 1% (wt/wt, norharmane/filter paper). Treatment and controls were randomly assigned to the right or left sides of the filter paper, and the filter paper was allowed to dry for 24 hours before testing. The dry filter paper was placed into a tub containing 100 termites, and feeding was evaluated 3 days later using a semi-quantitative scale (0, +, ++). Feeding bioassays with each concentration were replicated 5 times.

Norharmane Accumulation Bioassay: These bioassays were prompted by incidental observation that *Reticulitermes* workers produce small fluorescent balls of paper when held in petri dish bioassay arenas. This experiment was conducted to test the hypothesis that termites show a behavioral response to norharmane. Bioassay arenas were prepared by lining Pyrex petri dishes with Anchor Steel Blue Seed Germination Blotter paper. Each blotter paper was treated with 5 μl aliquots of norharmane in methanol at two concentrations (5 and 50 μg/μl) and a methanol control. The blotter paper was allowed to dry for 24 hours and then moistened with distilled water. Groups of approximately 30 *R. tibialis* or *R. flavipes* termites (workers and soldiers) were introduced into the arenas and kept in the dark for 3 days. Arenas were then examined for the presence of fluorescent paper balls. The bioassay was replicated 3 times.

Results

Histological observations under UV light showed that norharmane was contained in the termite internal fluids and not in the cuticle. Further observations indicated that norharmane is mainly located in the hemolymph, because hemolymph removal caused the loss of all observable fluorescence in termite carcasses (Fig 3.1). Many of the termites were observed to exude hemolymph, usually through the distal segment of the antennae (Fig. 3.1). TLC analyses of termite hemolymph, visualized under UV light, showed one bright spot, R_f 0.48, and a spot of less intensity at the origin (Fig 3.2). GC/MS analyses confirmed that norharmane was present in the hemolymph.

When live termites were present in petri dish arenas, small fluorescent balls of paper were observed. These were presumably made from the hemolymph-soaked blotter paper surrounding termite cadavers, suggesting that norharmane may be a behavioral cue. Both *R. tibialis* and *R. flavipes* showed no obvious behavioral response to synthetic norharmane, however, when it was spotted onto blotter paper in a bioassay arena.

Endosymbionts from *R. tibialis* and *R. flavipes* were extracted and cultured on Sabouraud dextrose agar with 1% yeast extract media. Examination under UV light showed the media surrounding certain isolates became intensely fluorescent (blue in color) after 5 days of growth (Fig. 3.3). Observations of the plates showed evidence of multiple microorganisms. Subsequent transfer and plating allowed the preparation of relatively pure microbial cultures that produced the fluorescent chromophore. Methanol extraction of the fluorescent media followed by GC/MS analysis indicated that norharmane was present at 200 ng/g of media (Fig. 3.4).

Feeding bioassays on filter paper with *R. tibialis* showed no feeding differences for any of the concentrations tested. This indicates that norharmane is neither a feeding

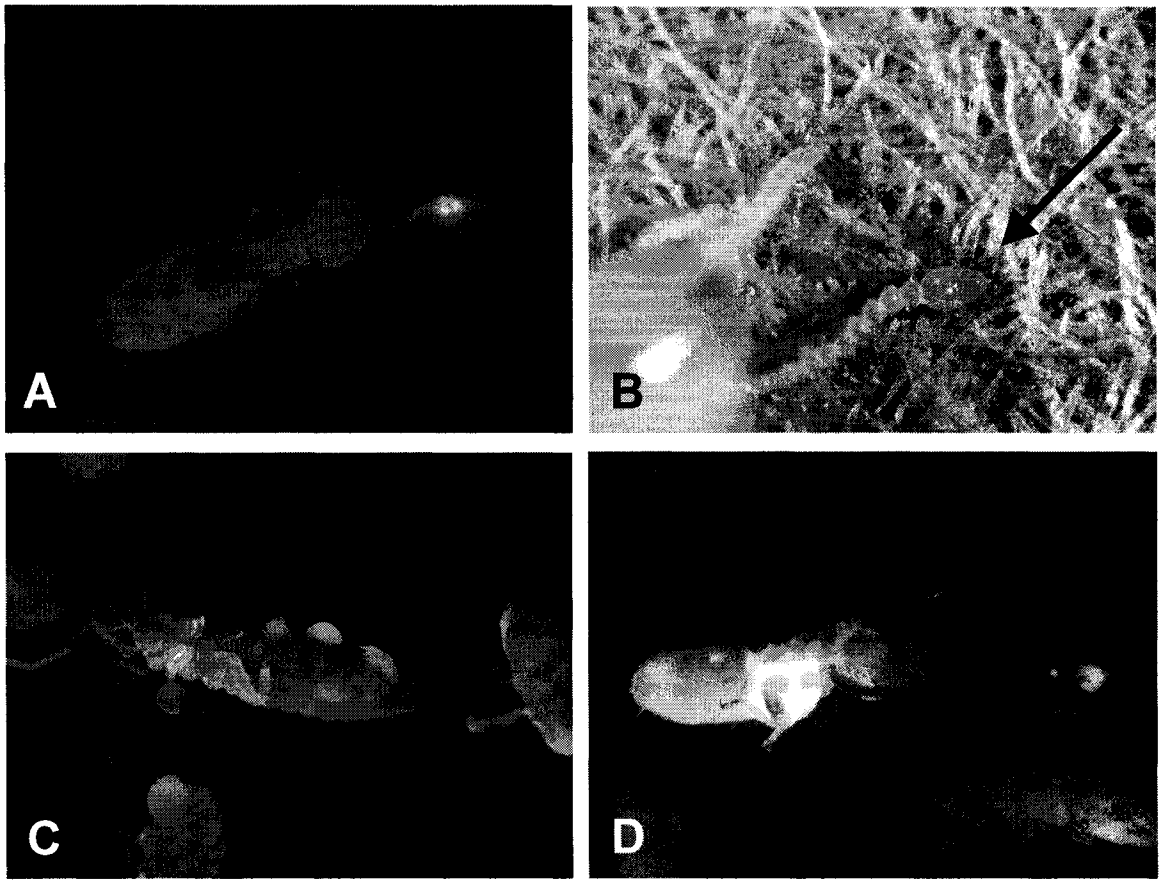

Figure 3.1 Hemolymph drainage from *Reticultermes tibialis*. Termite worker with swollen distal antennal segment, (A) under UV light, (B) magnified, under white light. (C) hemolymph exudation through thoracic and abdominal cuticle of a termite worker under UV light. (D) hemolymph exudation through thoracic and abdominal cuticle and distal antennal segment of a termite soldier under UV light.

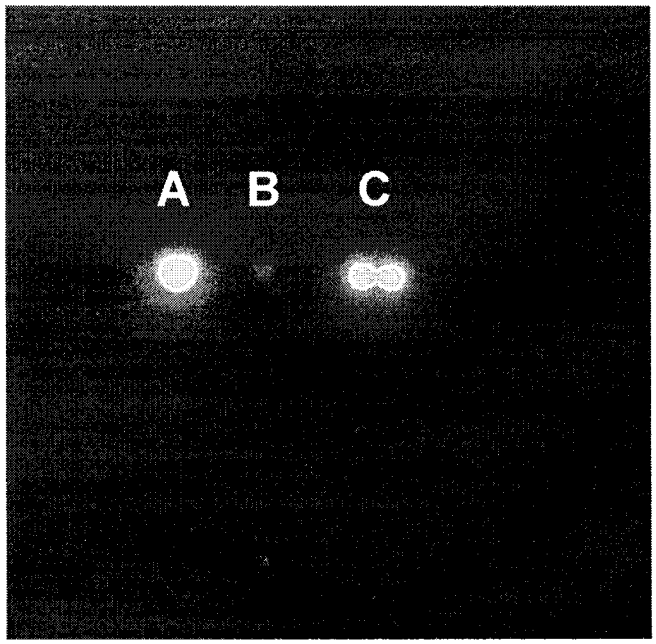

Figure 3.2 TLC of norharmane from *Reticultermes tibialis*. (A and C) synthetic norharmane, (B) hemolymph from *R. tibialis*. Silica gel TLC plates were developed with 75:15:10 n-butanol:water:formic acid and visualized by fluorescence under ultraviolet light.

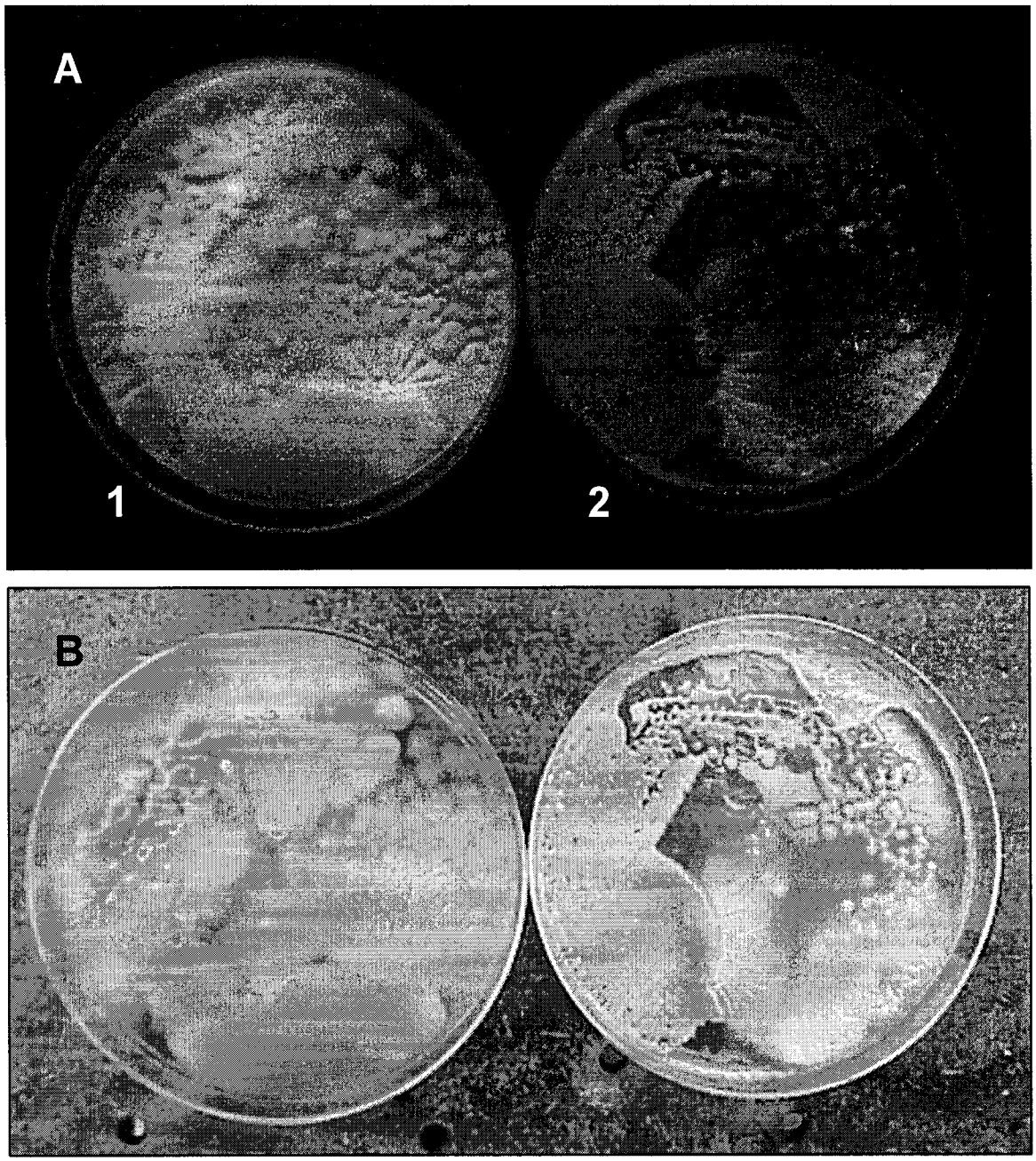

Figure 3.3 Whole-body endosymbiont extract from *Reticulitermes* termites. Microbial growth on Sabouraud dextrose agar with 1% yeast after 5 days. (A) *R. tibialis* (1) and *R. flavipes* (2) extracts shown under UV light. (B) The same plates shown under white light.

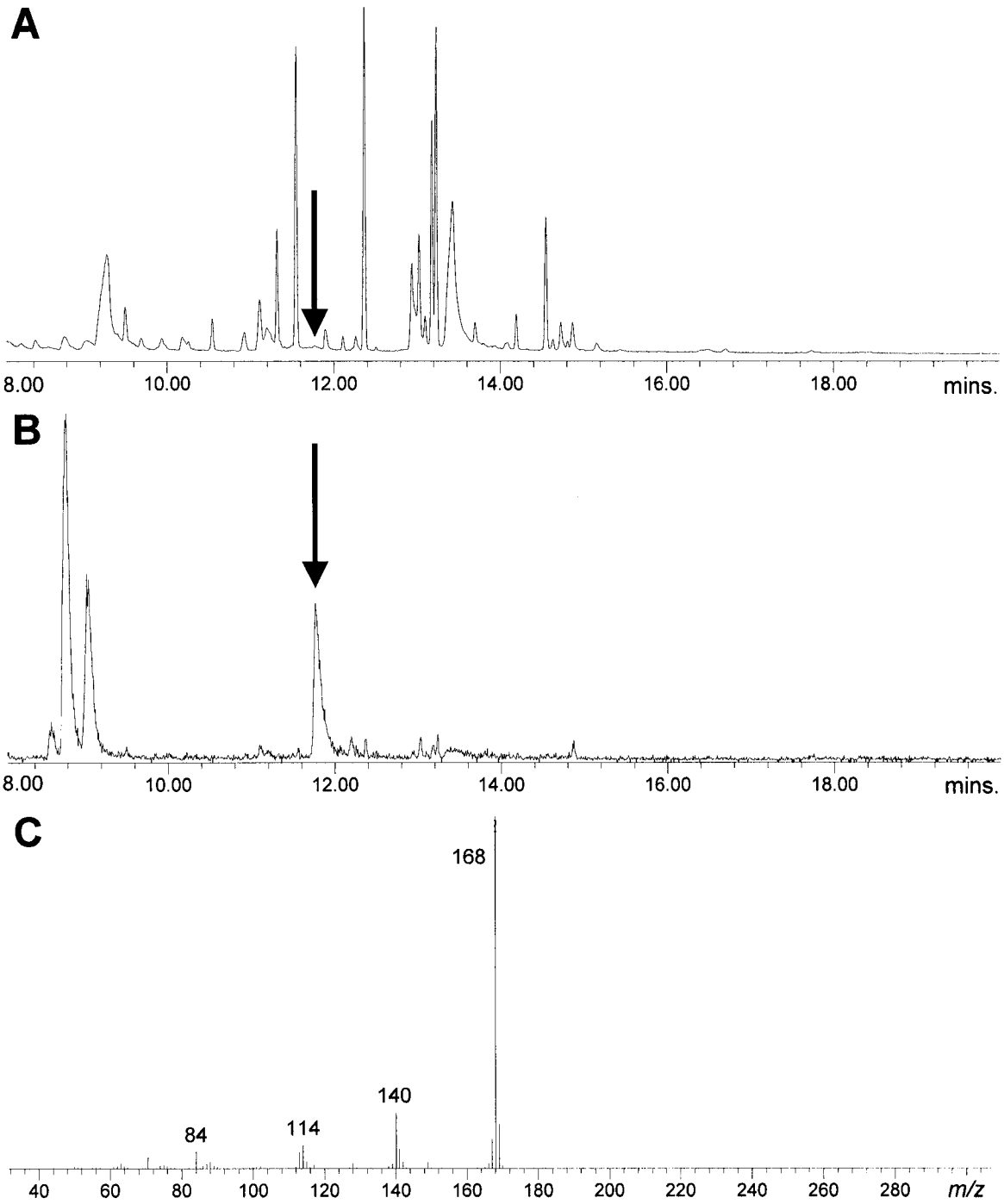

Figure 3.4 GC/MS analyses of termite microbial extract. Methanol extract of fluorescent media (arrow indicates norharmane peak at 11.78 minutes), (A) TIC chromatograph, (B) Extracted ion chromatograph (*m/z* 168). (C) Mass spectrum of 11.78 minute peak.

stimulant nor a feeding deterrent when tested at levels up to 1% (wt/wt norharmane/filter paper).

Discussion

As indicated by GC/MS analyses of metabolites from endosymbionts cultured in isolation from the insect, microorganisms biosynthesize norharmane in *R. tibialis* and *R. flavipes* termites. The importance of microorganisms in termite nutrition and evolution have been convincingly demonstrated (Abe et al. 2000, Cleveland 1924, Eutick et al. 1978, Veivers et al. 1983). Termites interact with a broad diversity of symbionts with representatives from the Archaea (methanogens), Eubacteria (bacteria) and Eucarya (protozoa and fungi) domains (Bignell 2000).

Prokaryotes are the most diverse group of termite endosymbiotic microorganisms (Breznak 2000). They include methanogens (Kudo et al. 1998, Leadbetter and Breznak 1996), spirochetes (Breznak 2002), *Proteobacteria*, representatives of the *Bacteroides* and low G+C Gram positive groups as well as other Eubacteria (Ohkuma and Kudo 1996). Prokaryotes occur in the termite gut, where they are most numerous (Breznak 2000), and are also intracellular symbionts (Bandi and Sacchi 2000). Appreciation of the importance of prokaryotes to termites has grown to the point were they are considered more important than interactions with protozoa (Bignell 2000). Termites are reliant on prokaryotes for carbon elimination (Higashi et al. 1992) and nitrogen fixation (Benemann 1973, Breznak et al. 1973) to achieve a C/N ratio conducive to growth, and acetogenesis from carbon dioxide (Breznak 1994). Bacterial metabolism of cellulose (Breznak and

Brune 1994), hemicellulose (Schafer et al. 1996) and aromatic compounds (predominantly lignin) (Breznak and Brune 1994, Brune et al. 1995, Kuhnigk et al. 1994) and sulfate reduction by bacteria (Kuhnigk et al. 1996) have also been studied, but their importance is less clear. In general, prokaryotes and particularly bacteria are relatively easy to isolate from termites, although there are some taxa, such as spirochetes, that have not yet been cultured (Breznak 2000).

Flagellate protozoa are present in the intestinal tracts of all the lower termites, and they metabolize cellulose to CO_2 and acetate (Inoue et al. 2000), the main energy source for termite respiration (Odelson and Breznak 1983). However, the exact nature of this interaction is unclear, because termites are now known to possess their own cellulases and can degrade at least a part of the cellulose they ingest in the absence of endosymbionts (Martin 1991, Rouland et al. 1988, Slaytor 1992, Watanabe et al. 1998). The relationship is further complicated by symbioses between protozoa and prokaryotes (Breznak 2000, Lee et al. 1987, Messer and Lee 1989, Odelson and Breznak 1985, Tamm 1982). Termite gut protozoa are difficult to culture due to their nutritional requirements (Berchtold et al. 1995, Odelson and Breznak 1985, Yamin 1978) and it is unlikely that they would have grown effectively under the conditions used in the present study. Therefore, protozoa are probably not the source on norharmane in the media cultures.

Fungal/termite interactions that cause the largest ecological impact are seen in fungus-cultivation by higher termites, particularly the subfamily Macrotermitinae, which cultivate exosymbiotic fungi of the genus *Termitomyces* (Rouland-Lefèvre 2000). In lower termites, fungal associations benefit termites in resource discovery, consumption and increased nutritional value (Rouland-Lefèvre 2000). Nutritional advantages are also

derived from fungal cellulases ingested from some food sources (Martin 1991). Not all fungal interactions are beneficial for termites. Due to the conditions in the natural habitats of subterranean termites, fungi represent a persistent disease risk (Blackwell and Rossi 1986, Grace and Zoberi 1992, Lutikova 1990, Zoberi 1995, Zoberi and Grace 1990a, Zoberi and Grace 1990b). Termite/fungal interactions are largely external (Rouland-Lefèvre 2000) so the surface sterilization of *Reticulitermes* likely limits the presence of fungi in termite extracts and subsequent media cultures, suggesting that fungi are also unlikely to be the source of norharmane in the endosymbionts cultures.

Norharmane has shown potent toxicity against a range of organisms (Bourke et al. 1992, de Meester 1995, Quetinleclercq et al. 1995, Yomosa et al. 1987) and may play a defensive role in termite biology (Siderhurst 2004a). *Streptomyces* spp. exosymbionts associated with attine ants produce antimicrobial compounds which are used to combat microbial pathogens of their fungal crop (Currie et al. 1999, Currie 2001, Poulsen et al. 2002) and a similar symbiosis has been suggested to for termites (Mueller and Gerardo 2002). *Streptomyces* bacteria produce multiple antimicrobial compounds, of interest ecologically and as a source of new drugs for use in human health (Paradkar et al. 2003). Bacteria in the *Streptomyces* genus belong to the Actinomycetes and are among the most numerous and ubiquitous soil bacteria (Holt et al. 1994). Bacteria feature prominently among the symbiotic intestinal microflora of termites and norharmane and its methylated analog harmane have been isolated from two genera of Actinomycetes (Arai et al. 1976, Yomosa et al. 1987). Actinomycetes are known components of the termite gut (Schafer et al. 1996) and would have had ample opportunity to coevolve with termites. Precedence for β-carboline production by an endosymbiont has been found in a tunicate-

associated bacterium (Aassila et al. 2003). Production of the antimicrobial compound norharmane by endosymbionts may demonstrate a biological role of termite endosymbionts other than nutrition.

Although we have established that termite endosymbionts produce norharmane, the identification of the particular microorganism involved was not attempted in this study. However, the only known instances of norharmane production in bacteria, fungi or flagellates are from the Actinomycetes bacteria, *Nocardia* sp. and *S. lavendulae* (Arai et al. 1976, Yomosa et al. 1987). Given this and the fact that Actinomycetes, and *Streptomyces* spp., are components of the termite gut biota (Schafer et al. 1996) there is a strong possibility that Actinomycetes bacteria are the source of termite norharmane.

The endosymbiotic origin of norharmane in termites contrasts strongly with the biosynthetic origin of norharmane in scorpions, the other arthropod group from which norharmane is known. Norharmane, the predominant fluorescent chromophore responsible for whole-body termite fluorescence under ultraviolet light, occur mainly in hemolymph. In contrast, scorpions contain norharmane in the epicuticular layer of their exoskeleton (Pavan 1954a, Pavan 1954b), where it is believed to be a by-product of cuticular sclerotization (Stachel et al. 1999). This difference in physiological distribution, coupled with the minimal sclerotization of *Reticulitermes* workers, suggests that scorpions and termites may differ in the biosynthetic origin of norharmane.

Termites are known to prefer certain woody species, but none of them are known to contain norharmane. Our observations that norharmane is not a feeding stimulant for *R. tibialis*, further indicate that termites do not actually obtain norharmane from their diet. While the present study suggests that endosymbionts are the biosynthetic origin of

norharmane in termites, it is possible that termites acquire norharmane from multiple sources. Precedence for defensive compounds with multiple origins is seen in the burnet moth, *Zygaena trifolii*, which biosynthesizes cyanogenic glycosides for defensive (Wray et al. 1983). These compounds are also sequestered from its host plant, *Lotus corniculatus* (Nahrstedt and Davis 1986). The moth may have been pre-adapted to deal with the toxic cyanogenic glycosides, which would have facilitated the development of the enzymatic systems needed to biosynthesize the compound independently.

The likely precursor for norharmane endosymbiont biosynthesis in termites is tryptophan. Feeding experiments with radio-labeled tryptophan in a number of plants have demonstrated that it is the biosynthetic origin of several simple β-carboline alkaloids related to norharmane (Allen and Holmstedt 1980, Liljegre 1968, Nettlesh and Slaytor 1974, Odonovan et al. 1976, Odonovan and Kenneall 1967, Slaytor and McFarlan 1968, Stolle and Groger 1968). Norharmane is a pyrolysis product of tryptophan and has been isolated from cooked foods that contain amino acids and/or protein (de Meester 1995). Termite dietary sources contain low amounts of amino acids or other nitrogenous compounds (Cowling and Merrill 1966, La Fage and Nutting 1978), and termites employ several strategies to address this deficiency. Feeding preferences for amino acids (Chen and Henderson 1996) increase amino acid uptake when encountered in a food source (Hungate 1941). Nitrogen-fixation by endosymbionts increase the amount of usable nitrogen for termites (Breznak 2000), and termites biosynthesize amino acids from acetate even with a reduced number of gut flagellate species (Mauldin et al. 1978).

The mechanism and possible biological significance of termite carcasses losing hemolymph through the distal segment of the antennae are as yet unresolved. The distal

segment of the antennae is likely a weak point in the insect cuticle due to the presence of a large number of cuticular-embedded receptors (Chapman 1998). Termites that exuded hemolymph were kept under moist conditions, and osmotic pressure could cause the swelling and eventual rupture the distal antennal segment. It is possible that this facilitates the use of norharmane, after the death of the individual termite, by other members of the colony. Norharmane has antimicrobial activity (Quetinleclercq et al. 1995, Siderhurst 2004a) and it might have defensive role in reducing the microbial pressure associated with termite carcasses. Hemolymph exudation may be analogous to reflexive bleeding, which is a defensive strategy employed by a number of insects (Blum 1981).

Our observations that termites produce fluorescent paper balls supports the suggestion that hemolymph exudation has a defensive role, as the balls may be used for microbial suppression within the colony. Termites line their tunnels with frass, which has shown antimicrobial activity (Rosengaus et al. 1998). Norharmane has been detected in *Reticulitermes* frass and this may be the active antimicrobial agent. *R. tibialis* and *R. flavipes* showed no behavioral response to synthetic norharmane spotted in bioassay arenas. However, chemical cues other than norharmane may help termites recognize hemolymph. Grasshoppers use free fatty acids as chemical cues for recognizing grasshopper cadavers, and termites may use similar cues (Bomar and Lockwood 1994a, Bomar and Lockwood 1994b).

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CHAPTER 4

ANTIMICROBIAL ACTIVITY OF THE TERMITE ALKALOID NORHARMANE AGAINST THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* (METSCHNIKOFF)

Introduction

The soil habitat and social behavior of termites allow rapid and easy transmission of pathogens among individuals within a colony (Rosengaus et al. 2000b, Rosengaus and Traniello 1997). Consequently, subterranean termites must contend with substantial disease pressure from bacteria (Castilhos-Fortes et al. 2002, Connick et al. 2001, Osbrink et al. 2001), fungi (Blackwell and Rossi 1986, Grace and Zoberi 1992, Lutikova 1990, Zoberi 1995, Zoberi and Grace 1990a, Zoberi and Grace 1990b), viruses (Alfazairy and Hassan 1988, Alfazairy and Hassan 1993) and other microorganisms. They must also contend with parasites such as nematodes (Epsky and Capinera 1988, Georgis et al. 1982, Massey 1971, Mauldin and Beal 1989, Nguyen and Smart 1994, Rouland et al. 1996, Wang et al. 2002a, Wang et al. 2002b, Wu et al. 1991) and mites (Phillipsen and Coppel 1977, Wang et al. 2002b). In ants, microbial pressure is countered by specialized exocrine glands, which produce an array of antimicrobial compounds (Attygalle et al. 1989, Attygalle and Morgan 1984, Beattie et al. 1985, Beattie et al. 1986, Hölldobler and Wilson 1990, Maschwitz et al. 1970). Termites lack analogous exocrine glands that are specificly dedicated to antimicrobial production (Noirot 1969) and instead rely on a diverse range of strategies such as behavioral responses to disease agents (Rath 2000), immune responses (Lamberty et al. 2001, Traniello et al. 2002), biochemical mechanisms (Lamberty et al. 2001, Maschwitz and Tho 1974, Olagbemiro et al. 1988, Rosengaus et al. 2000a), and symbiosis with protective fungi (Matsuura et al. 2000). These strategies have been reported from limited taxa (Rosengaus et al. 1998a), and are often limited to particular castes such as soldiers (Rosengaus et al. 2000a) or alates (Lamberty et al.

2001). Antimicrobial defenses with wide caste and phylogenetic distributions are yet to be reported.

The alkaloid norharmane has been isolated from *Reticulitermes tibialis* Banks, *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) and is the major component in termite fluorescence under ultraviolet light (Siderhurst 2004b). Norharmane is a phototoxin, and ultraviolet light exposure causes high autophototoxic mortality in *Reticulitermes* termites, suggesting novel pest control possibilities (Siderhurst 2004c). *Reticulitermes* termites are negatively phototactic to ultraviolet light, which may help to minimize autophototoxicity (Siderhurst 2004c). Norharmane in termites is contained in the hemolymph and is produced by endosymbionts, although the taxa and locations of these microorganism(s) are not yet known (Siderhurst 2004a).

The antimicrobial activity of norharmane suggests that it could be a critical component in termite disease resistance. Norharmane is the simplest of the ß-carboline alkaloids, which have a wide distribution in the plant kingdom (Allen and Holmstedt 1980) and occur with lower frequency in other taxa. The distribution of ß-carbolines is thought to reflect their broad spectrum of toxicity and phototoxicity against a variety of organisms (Allen and Holmstedt 1980). Norharmane has shown toxicity against fungi (Quetinleclercq et al. 1995) and plant seedlings (Yomosa et al. 1987), causes SOS responses and frame shift mutations in bacteria (Oda et al. 1987, Oda et al. 1988), sublethal nervous effects in mammals (Bourke et al. 1992), inhibition of cytochrome P_{450} (Nii 2003) and broad mutagenic/co-mutagenic properties (de Meester 1995). Other simple β-carbolines have shown toxicity toward *E. coli* (McKenna and Towers 1981) and trypanosomes (Rivas et al. 1999). In addition, norharmane exhibits potent phototoxic

activity against bacteria (Larson et al. 1988), insects (Larson et al. 1988) and eukaryotic cells (Towers and Abramowski 1983). Other ß-carbolines have also exhibited phototoxicity towards viruses (Hudson et al. 1986) and fungi (McKenna and Towers 1981), although phototoxic activity is likely precluded in the subterranean habitats of most termites (Schober and Lohmannsroben 2000).

Directly testing the possible role of norharmane in disease resistance in termites has proved problematic, because termites that lack norharmane have not been found and techniques that selectively remove norharmane from living termites have proven elusive. Although testing disease resistance in bioassays with live termites is currently impractical, dead termites without norharmane can be prepared by UV exposure (Siderhurst 2004c), presumably by reaction of excited state norharmane with target bio-molecules, and these cadavers or their hemolymph can be challenged with appropriate pathogens.

We report here the activity of norharmane against a major biocontrol agent, the entomopathogenic fungus, *Metarhizium anisopliae* (Metschnikoff) and the broad caste (workers, soldiers and alates) and phylogenetic distribution (18 termite genera distributed among 4 families) of this defense. Norharmane was shown to be absent from closely related taxa.

Materials and Methods

Insects. *Reticulitermes tibialis* Banks termites were obtained from two separate sites, Central Plains Experimental Range USDA-ARS Rangeland Resources Research

Unit and a private ranch outside Nunn, Colorado. Species identification was confirmed by Dr. Boris C. Kondratieff, insect systematist at Colorado State University. The termite collection trap was a wooden frame (14 cm x 10.5 cm) filled with corrugated cardboard sheets (10 cm x 7 cm) with a wire screen on the top and bottom. Wooden fence posts around cattle and horse ranches in Colorado are frequently infested with *R. tibialis* (suggested by the late Frances Lechleitner, Colorado State University). Traps were buried in the soil 15 to 20 cm deep next to fence posts, with ~1 L of water added to moisten the surrounding soil, and this often attracted large numbers of termites within a week. Termites were then placed in plastic tubs (Rubbermaid, 4 L) containing soil (20% moisture wt/wt) and stored in the laboratory at room temperature. *Reticulitermes flavipes* were obtained from Laurel, Mississippi. *Mantis religiosa* L. (European mantis), *Melanoplus* sp. (grasshopper) and *Periplaneta americana* (L.) (American cockroach) were collected in Fort Collins, Colorado. *Acheta domesticus* (L.) (house cricket) were purchased in Fort Collins, Colorado (Petco Animal Supplies, Inc., San Diego, California). *Cryptocercus punctulatus* Scudder were obtained from Dr. Timothy Judd, Southern Missouri University.

Alcohol samples from *Anoplotermes fumosus* (Hagen) (alates), *Amitermes wheeleri* (Desneux) (alates), *Cryptotermes brevis* (Walker) (alates), *Coptotermes formosanus* Shiraki (alates), *Heterotermes* sp. (soldiers and workers), *Incisitermes minor* (Hagen) (alates), *Kalotermes approximatus* Snyder (alates), *Marginitermes hubbardi* (Banks) (alates), *Microcerotermes* sp. (alates), *Nasutitermes costalis* (Holmgren) (alates), *Neotermes castaneus* (Burmeister) (alates and soldiers), *Paraneotermes simplicicornis* Banks (soldiers), *Prorhinotermes simplex* (Hagen) (alates), *Pterotermes occidentis*

(Walker) (alates), *Tenuirostritermes cinereus* (Buckley) (alates, nasutes and workers) and *Zootermopsis laticeps* (Banks) (soldiers and nymphs) termite specimens were obtained from the Frances Lechleitner Termite Flight and Distribution Survey collection, housed in the C. P. Gillette Museum of Arthropod Biodiversity, Colorado State University.

General Experimental Procedures. GC/MS analysis was performed on a Hewlett-Packard Series II gas chromatograph interfaced with a Hewlett-Packard 5971 Series Mass Selective Detector equipped with an Alltech Econo-Cap EC-1 column (30 m x 0.25 mm ID 0.25 μm film thickness). The temperature program was from 120 °C to 300 °C at 10 °C/min with a 1 min start delay. TLC was performed on pre-coated high performance Silica Gel 60 TLC plates (Alltech, Deerfield, Illinois). TLC plates were developed using a 75:15:10 n-butanol:water:formic acid solvent system (Stachel et al. 1999) and visualized by fluorescence under UV light. The UV source for TLC visualization and termite irradiation was a General Electric UV lamp (model 23301, 120 VAC, 60 Hz, 16 W) equipped with a GE FL15T8-black light bulb (output 300 – 400 nm). Ultraviolet levels in termite bioassays were monitored using an Eppley Ultraviolet Radiometer (290-385 nm). All solvents were HPLC grade except those used for spectroscopy, which were spectroscopic grade (Sigma-Aldrich, St. Louis, MO).

Qualitative GC/MS Analysis: Alcohol samples ranging in size from 1 to 20 ml were removed from museum specimens and evaporated to apparent dryness with a nitrogen stream. The residue was adsorbed onto pre-packed High Flow C-18 Extract-Clean solid phase extraction (SPE) columns (Alltech, Deerfield, Illinois) and eluted sequentially with water, water/MeOH and MeOH. The MeOH fractions were evaporated to apparent dryness with a nitrogen stream and then re-dissolved in a small volume (10-

100 µl) of MeOH for GC/MS and TLC analysis. Non-isopteran insects were prepared by grinding in MeOH and then sonicated (Disontegrator System Thirty sonicator, Ultrasound Industries, Plainview, New York) for 3 hrs. followed by centrifugation to remove visible tissue (International Clinical Centrifuge model CL, International Equipment Co., Needham, Massachusetts). The MeOH solution was removed and filtered before being evaporated to apparent dryness with a nitrogen stream and then re-dissolved in a small volume (10-100 µl) of MeOH for GC/MS and TLC analysis. For each sample a combination of TIC and extracted ion chromatographs (*m/z* 168, HP Standalone Data Analysis program), GC traces and TLC plates were examined to determine the presence or absence of norharmane.

Fungal Spore Suspension Preparation: Freeze-dried *M. anisopliae* var. *anisopliae* spores (ATCC 90448, media 325) were obtained from the American Type Culture Collection (Manassas, Virginia). The spores were rehydrated in distilled sterile water for 24 hours at room temperature and then inoculated onto plates of Sabouraud dextrose agar with 1% yeast extract (mycological peptone, 10.0 g/L; dextrose, 40.0 g/L; agar, 15.0 g/L; yeast extract, 1.0 g/L) (Reeves 2002). Fungal spore preparations were made using the methods of Rosengaus and Traniello (1997). Fungal suspensions were prepared by washing sporulated plates with a 0.1% Tween 80 (Aldrich, St. Louis, Missouri) solution in distilled sterile water. Spore counts were made with a Bright-line hemocytometer (Hausser Scientific, Horsham, Pennsylvania) under 400X magnification (Nikon Eclipse E400 stereomicroscope) followed by appropriate dilution to give fungal suspensions with an approximate spore concentrations of 1×10^4 and 1×10^6 spores/ml. Fungal suspensions were used immediately after preparation.

Fungal Inhibition Tests: Tests with synthetic norharmane were conducted using sterile Whatman #2 filter paper disks (12.5 mm dia., 13 mg) (Whatman International Ltd., Maidstone, England), which were treated with 20 µl aliquots of norharmane solutions in methanol using a Hamilton syringe (100 µl). A hundred-fold dilution series was used (10 ng/ml, 1 µg/ml, 100 µg/ml and 10 mg/ml) giving final concentration of norharmane on paper of 0.015 µg/g, 1.5 µg/g, 150 µg/g and 15,000 µg/g. MeOH treated filter disks were used as negative controls. The filter paper disks were allowed to dry thoroughly before being applied to Sabouraud dextrose agar with 1% yeast extract inoculated with 1 ml of a fungal spore suspension (1×10^4 or 1×10^6 spores/ml). Spore suspensions were spread with sterile glass beads and sterile technique was used throughout. Cultures were incubated at room temperature and results were recorded after 5 days. A clear zone of inhibition surrounding a treatment spot was scored as a positive result (+) with inhibition zones greater then 10 mm scored as (++) and no inhibition scored as a negative result (-).

Tests with termite hemolymph were conducted by applying hemolymph directly to plates treated with fungal spores (1×10^4 spores/ml). Groups of approximately 100 *R. flavipes* or *R. tibialis* termites consisting of both workers and soldiers were either irradiated with UV light for 6 hours to remove norharmane (NH-) or kept in the dark for 6 hours (NH+), which allowed the retention of norharmane at normal levels (~1 µg/g). Termites were irradiated in Pyrex petri dishes lined with Anchor Steel Blue Seed Germination Blotter paper moistened with distilled water. The UV source was positioned so that the light intensity reaching the termites was 60 W/m^2 as measured by radiophotometer. Both NH+ and NH- groups were frozen after treatment, ground and

then centrifuged for 15 min. The light yellow liquid that collected in the centrifuge tubes was removed using a microcapillary tube and applied to the agar plates. Cultures were incubated at room temperature and results were recorded after 5 days. Zones of inhibition were scored as above.

Tests with dead termites were conducted with carcasses placed directly on plates treated with fungal spores (1 x 10^4 spores/ml). Groups of *R. flavipes* or *R. tibialis* termites consisting of both workers and soldiers were either treated with UV light as above for 6 hours (NH-) or kept in the dark for 6 hours (NH+). Both groups were frozen after treatment and 5 - 20 whole carcasses were applied to the agar plates. Cultures were incubated at room temperature with observations recorded daily.

Results

A dilution series of synthetic norharmane on filter paper showed inhibition of *M. anisopliae* (1 x 10^4 spores/ml) at 1.5 μg/g (norharmane/filter paper), tested in the dark to preclude phototoxic effects that would not be expected to occur in a subterranean termite colony (Table 4.1). Fungal inhibition was most pronounced at the 15,000 μg/g treatment level, and lower concentrations exhibited uniform inhibition at 1.5 and 150 μg/g (Fig. 4.1). Norharmane showed no inhibition of *M. anisopliae* at 0.015 μg/g. At the higher spore concentration of 1 x 10^6 spores/ml, inhibition was only seen at the 150 and 15,000 μg/g norharmane level.

Termite hemolymph containing norharmane inhibited fungal growth (+) when challenged at 1 x 10^4 spores/ml (Table 4.2), but hemolymph from termites treated with

Table 4.1 Inhibition of *Metarhizium anisopliae* by synthetic norharmane. Fungal inhibition tests were incubated in the dark to preclude phototoxic effects. (+) = fungal inhibition, clear zone < 10 mm; (++) = fungal inhibition, clear zone > 10 mm; (–) = no fungal inhibition.

	Norharmane conc. (µg/g)				
	0	0.015	1.5	150	15,000
Spore conc. (spores/ml)					
1×10^4	–	–	+	+	++
1×10^6	–	–	–	+	++

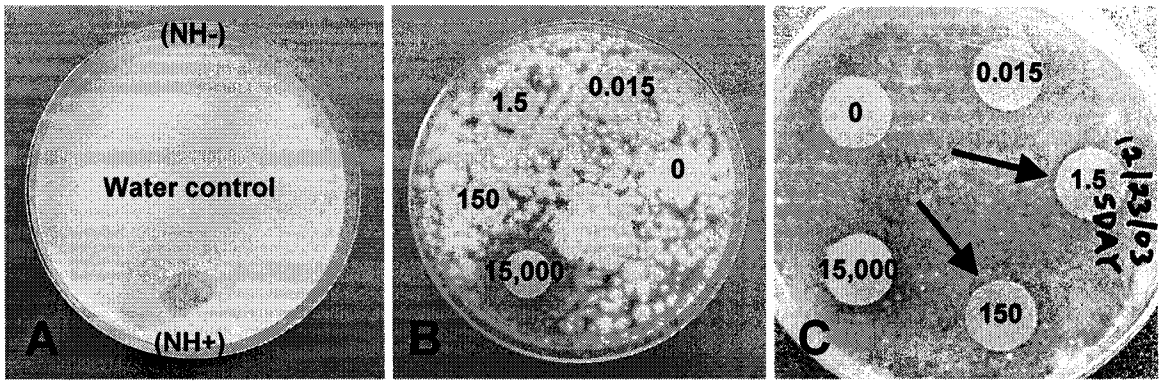

Figure 4.1 Fungal inhibition by norharmane. (A) *Reticultermes tibialis* hemolymph challenged with 1 x 10^4 spores/ml. (B) Synthetic norharmane (μg/g) challenged with *Metarhizium anisopliae* at 1 x 10^6 spores/ml. (C) Synthetic norharmane (μg/g) challenged with *M. anisopliae* at 1 x 10^4 spores/ml. Arrows indicates thin zone of inhibition. NH– = no norharmane, UV treated; NH+ = norharmane, no UV treatment.

Table 4.2 Inhibition of *Metarhizium anisopliae* by termite hemolymph. Fungal inhibition tests were incubated in the dark to preclude phototoxic effects. (+) = fungal inhibition, clear zone < 10 mm; (++) = fungal inhibition, clear zone > 10 mm; (–) = no fungal inhibition; NH– = no norharmane, UV treated; NH+ = norharmane, no UV treatment.

	Treatment		
	H_2O	NH–	NH+
R. tibialis	–	–	+
R. flavipes	–	–	+

UV light to remove norharmane did not inhibit fungal growth (–) (Fig. 4.1). No fungal inhibition was observed when whole termites, treated with UV (lacking norharmane) or controls (containing norharmane), were exposed to *M. anisopliae* at 1 x 10^4 spores/ml.

Chemical analysis of 18 genera from four of the seven families in the Isoptera (termites) showed that norharmane was present in all termite species analyzed (Fig. 4.2). In contrast, norharmane was absent in chemical analyses of species from five phylogenetically related taxa, including wood roaches (*C. punctulatus*), roaches (*P. americana*), mantids (*M. religiosa*), crickets (*A. domesticus*) and grasshoppers (*Melanoplus* sp.).

Discussion

The inhibition *M. anisopliae* by ecologically relevant concentrations of norharmane suggests that it may contribute to disease resistance in termites, consistent with its antimicrobial activity and role as a chemical defense in other organisms (Bourke et al. 1992, Yomosa et al. 1987). The entomopathogenic fungus *M. anisopliae* is a leading biological control agent for termites and other insects (Grace 2003) and has been used to investigate disease resistance mechanisms in termites (Rosengaus et al. 1998b, Rosengaus et al. 1998a, Rosengaus et al. 1999, Rosengaus et al. 2000a, Rosengaus et al. 2000b, Rosengaus and Traniello 1997, Rosengaus and Traniello 2001, Traniello et al. 2002). The minimum concentration of norharmane that showed inhibition of *M. anisopliae* was within an order of magnitude of previously reported inhibitory values for other fungal species (Quetinleclercq et al. 1995). In addition, this concentration is

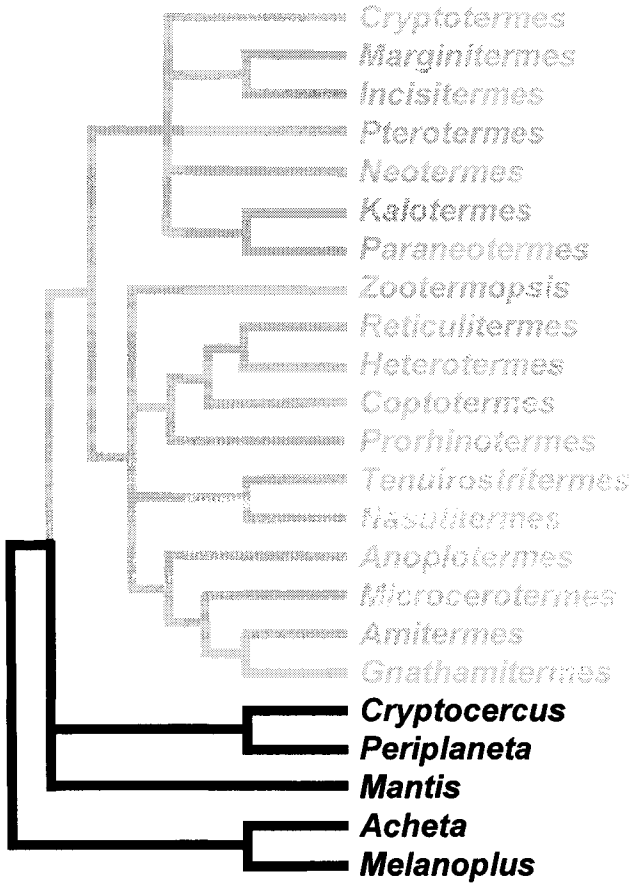

Figure 4.2 Phylogenetic distribution of norharmane in Isopteran genera and related taxa. Genera containing norharmane are shown in gray while those not containing norharmane are shown in black. Cladogram adapted from Krishna (1970), Thorne and Carpenter (1992) and Wheeler et al. (2001).

comparable to the minimum norharmane levels that show toxicity in bacteria (Oda et al. 1988) and plants (Yomosa et al. 1987), and phototoxicity in eukaryotic cells (Towers and Abramowski 1983).

Termite hemolymph inhibition of *M. anisopliae* suggests that norharmane is active at ecologically relevant concentrations. However, there may be other antimicrobial compounds present in the hemolymph that act alone or synergistically with norharmane (Sannasi 1968), and these may be sensitive to UV light. Hemolymph antimicrobial activity is then suggestive but not definitive. The termite cadaver bioassays failed to show obvious differences between NH+ and NH– termites. This was not unexpected because cadavers are particularly susceptible to attack and other known components of antimicrobial defense, such as behavioral responses (Rath 2000), were effectively removed.

The clearest evidence for norharmane as a defensive compound against microbial pathogens comes from the fungal inhibition by synthetic norharmane at 1.5 μg/g. *Reticulitermes* termites contain approximately 1 μg/g of norharmane and this is largely restricted to the hemolymph (Siderhurst 2004a, Siderhurst 2004b). Hemolymph volumes for termite queens and hemolymph/body mass ratios from related species suggest that a reasonable estimate for a hemolymph percentage may be 15% of the total body weight (Bordereau 1976, Joly 1940, Jones 1977). The concentration of norharmane in termite hemolymph is therefore approximantely 10 μg/g, at which fungal inhibition has been shown in the present study and with other fungal species (Quetinleclercq et al. 1995). The activity of norharmane against the fungal pathogen at ecologically relevant

concentrations supports the hypothesis of norharmane playing an antimicrobial role in termites.

Norharmane was found in 18 genera from the four largest families in the Isoptera (Kambhampati and Eggleton 2000) and it is therefore the most widespread antimicrobial termite defense yet reported. While alcohol samples were mostly taken from termite alates, some storage vials included or exclusively contained other castes. In addition, norharmane was previously found in worker, soldier and alate termite castes from *R. tibialis* (Siderhurst 2004b). Norharmane is therefore a broadly distributed antimicrobial defense across castes as well as phylogenetically. The broad distribution of norharmane in termites and its absence from phylogenetically related taxa (Fig. 4.2), may also suggest a monophyletic origin for norharmane in termites. This absence may reflect a decreased pathogenic pressure in taxa that are not social or soil dwelling. The absence of norharmane is particularly striking in the wood roach, *C. punctulatus*, which likely face a disease pressure similar to termites. Wood roaches share similarities with termites in sociality, habitat (decaying wood), diet (wood) and digestion (protozoan assisted cellulose metabolism) and may be the Dictyopterans most closely related to termites (Eggleton 2001).

Previous reports of antimicrobial defense strategies in termites are in general limited phylogenetically and in caste distribution. Reported disease defenses can be divided into three categories; behavioral responses, biochemical mechanisms and protection derived from symbiosis with protective fungi. Research with the dampwood termite, *Z. angusticollis* (Hagen), has shown that behavioral responses, such as vibratory displays on contacting *M. anisopliae* and grooming following exposure, play a critical

role in disease resistance (Rosengaus et al. 1998b). Grooming has also been implicated in *M. anisopliae* resistance in *R. speratus* Kolbe (Shimizu and Yamaji 2003) and *N. exitiosus* (Hill) (Rath 2000). Other behavioral responses include entombing and/or removal of dead or diseased nestmates (Kramm et al. 1982, Milner et al. 1998, Rath 2000), avoidance and enclosure of pathogen-infected areas (Ko et al. 1982, Milner and Staples 1996, Rosengaus et al. 1999) and use of antimicrobial feces in the construction of nest chambers and galleries (Rosengaus et al. 1998a). Behavioral strategies occur across castes and likely constitute a microbial defensive role in many termite species. In addition, their relative ecological importance has been easier to ascertain than biochemical mechanisms.

A range of biochemical mechanisms for disease resistance have been reported in termites and are more taxonomically and caste limited than behavioral strategies. Humoral immune responses have been shown in *Z. angusticollis*, and in *Pseudacanthotermes spiniger* (Sjostedt). *Z. angusticollis* termites undergo physiological changes after exposure to sublethal doses of a microbial pathogen and not only show increased disease resistance but can transfer this resistance to unexposed individuals of the same colony. In *P. spiniger* two novel antimicrobial peptides have been isolated and characterized in response to septic injury. One peptide was found in hemocyte granules and salivary glands, and both peptides were present in bacterially challenged and unchallenged termite. Secretions from the multifunctional glands of Macrotermitine (Maschwitz and Tho 1974), *Odontotermes* (Batra and Batra 1979, Olagbemiro et al. 1988), *Nasutitermes* (Rosengaus et al. 2000a) and *Zootermopsis* species have been shown to inhibit microbial growth. In addition, antimicrobial activity has also been found in the

bodily fluids of *Anacanthotermes ahngerianus* Jacobson (Lutikova 1990, Lutikova and Judina 1996), *Microcerotermes cameroni* Snyder (Sannasi 1968), *Odontotermes redemanni* (Wasmann) (Sannasi and Rajulu 1967) and *R. lucifugus* Rossi (Lavie 1960). In some instances the active compounds have been identified while other reports provide limited or no information about the antimicrobial chemicals involved. Evidence of ecological relevance and/or biological assays are absent from many of these studies and further work is needed to determine the relative importance of these strategies.

Termites owe much of their evolutionary success to symbiotic relationships with microorganisms (Bignell 2000). A disease resistance component of this relationship has been discovered with the symbiotic *Fibularhizoctonia* sp. fungus (Matsuura et al. 2000). Sclerotia of *Fibularhizoctonia*, which are morphologically similar to termite eggs, are often piled with the eggs of *R. speratus*. When tended by workers sclerotia increase egg survival apparently by protecting eggs from pathogens, a defense that may be employed in other taxa. Endosymbiotic production of norharmane (Siderhurst 2004a) is a second example of microbial symbiont assistance in termite disease resistance.

The mechanism of ß-carboline toxicity in the absence of UV light is well studied but is still somewhat unclear. Studies with membrane-free phages suggest that DNA is the probable target molecule for many ß-carbolines (Towers and Hudson 1987). Consistent with this, DNA intercalation of norharmane (Nii 2003) and oxidative DNA damage caused by aminophenylnorharman formed from norharman and aniline are proposed mechanisms of toxicity (Masumura et al. 2003, Ohe et al. 2002, Ohnishi et al. 2001, Totsuka et al. 2002). In addition, broad mutagenic/co-mutagenic activity (de Meester 1995), inhibition of monoamine odixase (Heinz et al. 1996), cytochrome P_{450}

(Kuhnvelten 1993), nitric acid synthase (Lee et al. 2000) and GABA receptors in insect nervous systems (Bloomquist et al. 1997), all represent possible modes of toxic activity.

Tyrophagus mites and predatory pavement ants (*Tetramorium caespitum* L.) fed on diets containing norharmane showed no mortality in either species on exposure to UV light (Siderhurst 2004c). Longer-term experiments with attention to sublethal effects may well show some activity of norharmane toward parasites and predators of termites, but the toxicity appears to be too low to be an effective defense.

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CHAPTER 5

ULTRAVIOLET LIGHT INDUCED AUTOPHOTOTOXICITY AND NEGATIVE PHOTOTAXIS IN *RETICULITERMES TIBIALIS* BANKS, *RETICULITERMES FLAVIPES* (KOLLAR) AND *RETICULITERMES VIRGINICUS* (BANKS) (ISOPTERA: RHINOTERMITIDAE)

Introduction

The fluorescent alkaloid norharmane has been found in all termites so far analyzed (Siderhurst 2004a). Norharmane has antimicrobial activity against the entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) and is a critical defense component in countering the significant disease pressure experienced in termites' subterranean habitats (Siderhurst 2004a). This defensive mechanism seems to have both wide phylogenetic distribution as norharmane has been isolated from 18 termite genera distributed among 4 families (Siderhurst 2004a), and broad caste involvement, known from *Reticulitermes* workers, soldiers and alates (Siderhurst 2004c). Selective pressures are high for chemical defenses among microbes and many antimicrobial compounds have their biosynthetic origin in other microbial species. Termites possess a diverse range of intestinal (Bignell 2000) and intracellular (Bandi and Sacchi 2000) microfauna, which function mainly in a nutritional role, but have also been speculated to produce antimicrobial compounds (Mueller and Gerardo 2002). The GC/MS analysis of metabolites from endosymbionts isolated from *Reticulitermes* termite show that norharmane has its biosynthetic origin in a termite symbiont (Siderhurst 2004b). Defensive strategies do not come without costs, although these costs may be hidden or indirect. Defenses may be costly both in terms of resources diverted to defense and the risks associated with sequestering toxic defensive compounds.

Although norharmane and other ß-carbolines have received attention for their inherent toxicity (de Meester 1995), their ecological role has also been heavily studied with respect to their phototoxicity. Norharmane is known to exhibit potent phototoxic

activity against bacteria (Larson et al. 1988), insects (Larson et al. 1988) and eukaryotic cells (Towers and Abramowski 1983). Other ß-carbolines have also exhibited phototoxicity towards viruses (Hudson et al. 1986) and fungi (McKenna and Towers 1981). Even though UV levels are low in subterranean habitats (Schober and Lohmannsroben 2000), storing norharmane may make termites susceptible to UV-mediated phototoxic effects.

The role of norharmane as an antimicrobial defense in termites has already been demonstrated *in vitro* (Siderhurst 2004a), but a defensive role against invertebrate predators and parasites has not yet been investigated. Termites experience substantial predation pressure both in their nests and while foraging (Abe and Darlington 1985, Korb and Linsenmair 2002, Lepage 1981, Prestwich 1984, Sheppe 1970). Ants are among termite's most prominent invertebrate predators with some ants preying on termites opportunistically and some specializing on termite predation (Hölldobler and Wilson 1990). While not all interactions are antagonistic, termites and ants compete for ecological niches, and the interaction between the two groups of social insects has been referred to as a coevolutionary arms race (Hölldobler and Wilson 1990).

Mites associated with termites are poorly studied in relation to their abundance (Eickwort 1990). While other invertebrates such as nematodes are parasitic and have received attention for their potential use as biocontrol agents (Epsky and Capinera 1988, Georgis et al. 1982, Massey 1971, Mauldin and Beal 1989, Nguyen and Smart 1994, Rouland et al. 1996, Wang et al. 2002a, Wang et al. 2002b, Wu et al. 1991) the ecological impact of mites on termites is largely unknown. Most mites associated with termites are phoretic or saprophytic but instances of negative interactions have been

reported (Costa-Leonardo and Soares 1993, Eickwort 1990, Myles 2002, Phillipsen and Coppel 1977a, Phillipsen and Coppel 1977b, Wang et al. 2002b). We observed that mites associated with *Reticulitermes* termites became fluorescent after feeding on termite carcasses, which may indicate the transference of norharmane to the mites.

The objective of the present study was to investigate possible UV-mediated phototoxic effects in *Reticulitermes* termites due to the presence of norharmane and mechanisms to counter any possible negative effects. The phototoxic activity of norharmane as a possible defense against invertebrate parasites and predators was also addressed with mites and ants.

Materials and Methods

Insects and Mites. *Reticulitermes tibialis* Banks termites were obtained from two separate sites, Central Plains Experimental Range USDA-ARS Rangeland Resources Research Unit and a private ranch outside Nunn, Colorado. Species identification was confirmed by Dr. Boris C. Kondratieff, insect systematist at Colorado State University. The termite collection trap was a wooden frame (14 cm x 10.5 cm) filled with corrugated cardboard sheets (10 cm x 7 cm) with a wire screen on the top and bottom. Wooden fence posts around cattle and horse ranches in Colorado are frequently infested with *R. tibialis* (suggested to us by the late Frances Lechleitner, Colorado State University). Traps were buried in the soil 15 to 20 cm deep next to fence posts, with ~1 L of water added to moisten the surrounding soil, and this often attracted large numbers of termites within a week. Termites were then placed in plastic tubs (Rubbermaid, 4 L) containing

soil (20% moisture wt/wt) and stored in the laboratory at room temperature. *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) were obtained from Laurel, Mississippi.

Mites were removed from *R. flavipes* and *R. virginicus* laboratory colonies and cultured in Pyrex petri dishes. The petri dishes were lined with Anchor Steel Blue Seed Germination Blotter paper moistened with distilled water (Anchor Paper Co., St Paul, Minnesota). Frozen termites (*R. tibialis*) were added, as food items, at three-day intervals. The blotter paper was rewetted as needed to maintain consistent moisture levels. Gravid adults were removed to start new dish cultures at two-week intervals. Mites were identified as *Tyrophagus* sp. by John C. Moore, University of Northern Colorado. *Tetramorium caespitum* L. (pavement ants) were collected in Fort Collins, Colorado, and stored in the laboratory no more then 24 hours before use. Termites, ants and mites were transferred with a small paintbrush (#2, Cirrus 440 Round, Kolinsky sable, Winsor and Newton, England).

General Experimental Procedures. The HPLC system used for analysis consisted of a Spectra-Physics SP8800 ternary pump, Valco C6W sample loop injector (10 μl loop), and a Spectra-Physics Spectra 100 variable wavelength detector. The detection wavelength was 254 nm. An Econosphere C-18 column (250 cm x 4.6 mm) (Alltech, Deerfield, Illinois) was used for quantitative analysis. Separation was achieved with an isocratic 60% methanol:40% 50 mM ammonium acetate buffer (pH 8) solvent system (Stachel et al. 1999). TLC was performed on pre-coated high performance Silica Gel 60 TLC plates (Alltech, Deerfield, Illinois). TLC plates were developed using a 75:15:10 n-butanol:water:formic acid solvent system (Stachel et al. 1999) and visualized

by fluorescence under UV light. The UV source for observations of fluorescence and bioassays was a General Electric UV lamp (model 23301, 120 VAC, 60 Hz, 16 W) equipped with a GE FL15T8-black light bulb (output 300 – 400 nm). Ultraviolet levels in termite bioassays were monitored using an Eppley Ultraviolet Radiometer (290-385 nm). All solvents were HPLC grade except those used for spectroscopy, which were spectroscopic grade (Sigma-Aldrich, St. Louis, MO).

Termite UV Mortality Bioassay: This experiment was conducted to test the hypothesis that termites exposed to UV light would show higher levels of mortality than unexposed termites. Live *R. flavipes, R. virginicus* and *R. tibialis* termites were used in this bioassay. Groups of approximately 30 termites (workers and soldiers) were placed in Pyrex petri dishes lined with Anchor Steel Blue Seed Germination Blotter paper moistened with distilled water. They were then exposed to UV light for 24 hours, with UV light excluded from controls with pieces of cardboard. The UV source was positioned so that the light intensity reaching the termites was 60 W/m^2 for high intensity treatments or 30 W/m^2 for low intensity treatments as measured by radiophotometer. Direct sunlight UV levels were determined to be ~120 W/m^2 (summer morning, Fort Collins, CO). Mortality was recorded after 24 and/or 48 hours with termites scored as 'alive', 'dead' or 'dying'. Selected bioassay arenas were left intact following the 48-hour test period to qualitatively observe longer-term effects on treatment and control termites. Mites and ants were used in control experiments and were treated under the same UV conditions with the following adjustments. Ants were housed in plastic tubs (Rubbermaid, 414 ml) with modified lids to ensure they did not escape during the bioassay. Lids were modified by cutting a 10 cm hole and inserting a Pyrex petri dish lid,

which was secured with hot melt glue (Hobby Lobby, Oklahoma City). Modified lids had the same UV permeability as standard Pyrex petri dishes as measured by radiophotometer. Replicate numbers (N) were dependent on termite availability; 60 W/m^2 replicate numbers were 44 for *R. flavipes,* 26 for *R. tibialis* and 23 *R. virginicus*, 30 W/m^2 replicate numbers were 6 for *R. flavipes,* control replicates numbers were 25 for *R. flavipes,* 9 for *R. tibialis* and 12 *R. virginicus.*

Termite UV Phototaxis Bioassay: This experiment was conducted to test the hypothesis that termites exposed to UV light would show negative phototactic behavior. Live *R. flavipes, R. virginicus* and *R. tibialis* termites were used in this bioassay. Groups of approximately 30 termites (workers and soldiers) were placed in Pyrex petri dishes lined with Anchor Steel Blue Seed Germination Blotter paper moistened with distilled water. They were then exposed to UV light for 24 hours, with UV light excluded from half the petri dish with pieces of cardboard. The cardboard positions were alternated at 90^o orientations relative to the UV source to prevent bias across replications. The UV source was positioned so that the light intensity reaching the termites was 60 W/m^2 as measured by radiophotometer. The numbers of termites on each of the respective halves of the bioassay arenas were recorded after 24 hours. Replicate numbers were 16 for *R. tibialis* and 9 for *R. flavipes.*

Norharmane Transference and Phototoxicity Bioassays with Mites: Adult *Tyrophagus* mites were placed in Pyrex petri dishes lined with Anchor Steel Blue Seed Germination Blotter paper moistened with distilled water. Frozen termites (*R. tibialis*) with (NH+) or without (NH-) norharmane were added as the sole food source. Termite carcasses were prepared by irradiating groups of termites, consisting of both workers and

soldiers, either with UV light for 6 hours to remove norharmane (NH-) or kept in the dark for 6 hours with norharmane being retained (NH+). Mites were allowed to feed for 7 days before being exposed to UV light (60 W/m^2) for 48 hours. Mortality was recorded after 48 hours. The bioassay was replicated twice. Mites tested for norharmane presence were ground in methanol, the solution was filtered and blown to apparent dryness under a nitrogen stream. The sample was redissolved in 10 μl of methanol and the entire solution was injected into the HLPC system or spotted onto TLC plates for analysis.

Norharmane Feeding and Phototoxicity Bioassays with Ants: This experiment was conducted to test the hypothesis that ants fed on norharmane and sucrose would show higher levels of mortality than termites fed sucrose only, when both groups were exposed to UV light. Groups of approximately 100 ants were placed in plastic tubs with modified lids. Control treatments were provided 2 x 1 ml of a 20 % sucrose solution (aqueous) in 1 cm diameter plastic vial caps. Norharmane treatments were provided solutions of 20% sucrose/sat. norharmane (solubility 30 μg/ml) (Varela et al. 1995b) in the same manner. Ants from both groups were ground in methanol and examined under UV light to confirm that norharmane was ingested. Ants were allowed to feed for 7 days before being exposed to UV light (60 W/m^2) for 48 hours. Mortality was recorded after 48 hours. The bioassay was replicated 8 times for ants fed on norharmane and sucrose and 7 times for ant fed sucrose only.

Statistical Analysis. Tukey's HSD test was used to analyze UV-mediated mortality for termites, and ants fed on norharmane (SAS 2000). T-tests were used to analyze termite phototaxis response data, and UV-mediated mortality for ants used as negative controls for termite UV-mortality bioassay (SAS 2000). χ^2 tests were used to

analyze mortality for mites fed on termites and mites used as negative controls for termite UV-mortality bioassay (SAS 2000).

Results

All three *Reticulitermes* termite species displayed significantly ($P<0.001$) higher mortality, when exposed to 60 W/m^2 intensity UV light for 24 hours, than controls kept in the dark (Fig. 5.1). *Reticulitermes flavipes* displayed almost complete mortality when UV exposed with *R. virginicus* and *R. tibialis* showed slightly less susceptibility (Fig. 5.1). Individuals scored as 'dying' had significantly decreased mobility and generally died within 48 hrs of treatment while termites scored as 'living' showed high mobility and remained viable for several weeks following treatment. *Reticulitermes flavipes* exposed to 30 W/m^2 intensity UV light showed light mortality at 24 hours but showed levels of mortality comparable to the 60 W/m^2 treatment at 48 hours without additional UV treatment (UV treatment for 24 hours + 24 hours in the dark = 48 hour mortality) (Fig. 5.2). Termites exposed to UV light lost their fluorescence within hours of UV exposure (Fig. 5.3), but control termites showed no decrease in fluorescence. HPLC analysis of UV-treated termites revealed no detectable levels of norharmane.

In contrast to the high mortality recorded in *Reticulitermes* termites, no significant levels of mortality, on exposure to UV light, were shown with mites or ants (Fig. 5.4). The mites found with the termites showed no mortality when exposed to 60 W/m^2 UV light. Pavement ants also showed no UV-attributable mortality when exposed to 60 W/m^2 UV light.

Termites exposed to 60 W/m^2 UV light for 24 hours in half-covered petri dishes showed a significant (P<0.001) preference for the covered half of the dish (Figs. 5.5 & 5.6). Termite mortality was significantly (P<0.001) reduced in these tests compared to 60 W/m^2 and 30 W/m^2 direct exposure (Fig. 5.7).

The relationship of the mites found on both *R. flavipes* and *R. virginicus* to their termite hosts is unclear, but is most likely saprophagous. Mites were initially observed in termite bioassays feeding on dead termites. The mites were never observed while feeding on live termites and were only observed infrequently on live termites at all. Mites fed on termites containing norharmane (NH+) became fluorescent while those feeding on termites without norharmane (NH–) remained flat/dull under UV light (Fig. 5.8). The mites fluoresced a blue/green color under UV light, matching the fluorescence observed in termites. HPLC qualitative analyses show that the fluorescent mites contained norharmane while the flat/dull mites did not. TLC analysis of fluorescent mites showed a spot that fluoresced under UV light, with R_f 0.48 matching the R_f for norharmane. This spot was not present in non-fluorescing mites. An additional fluorescent spot of similar color to norharmane, with Rf 0.53, was also observed on the mite extract TLC plate. Observations of fluorescent mites indicate that the norharmane is located in defined internal areas, in structures that resemble fat bodies (Fig. 5.8). Forty-eight hour UV light exposure (60 W/m^2) did not cause any mortality in the fluorescent mites (Fig. 5.9) in contrast to the high mortality seen in termites. However, fluorescent mites were observed to lose their fluorescence after treatment as seen in termites. HPLC analyses show that mites that previously contained norharmane and fluoresced contained no norharmane after 24 hour UV exposure.

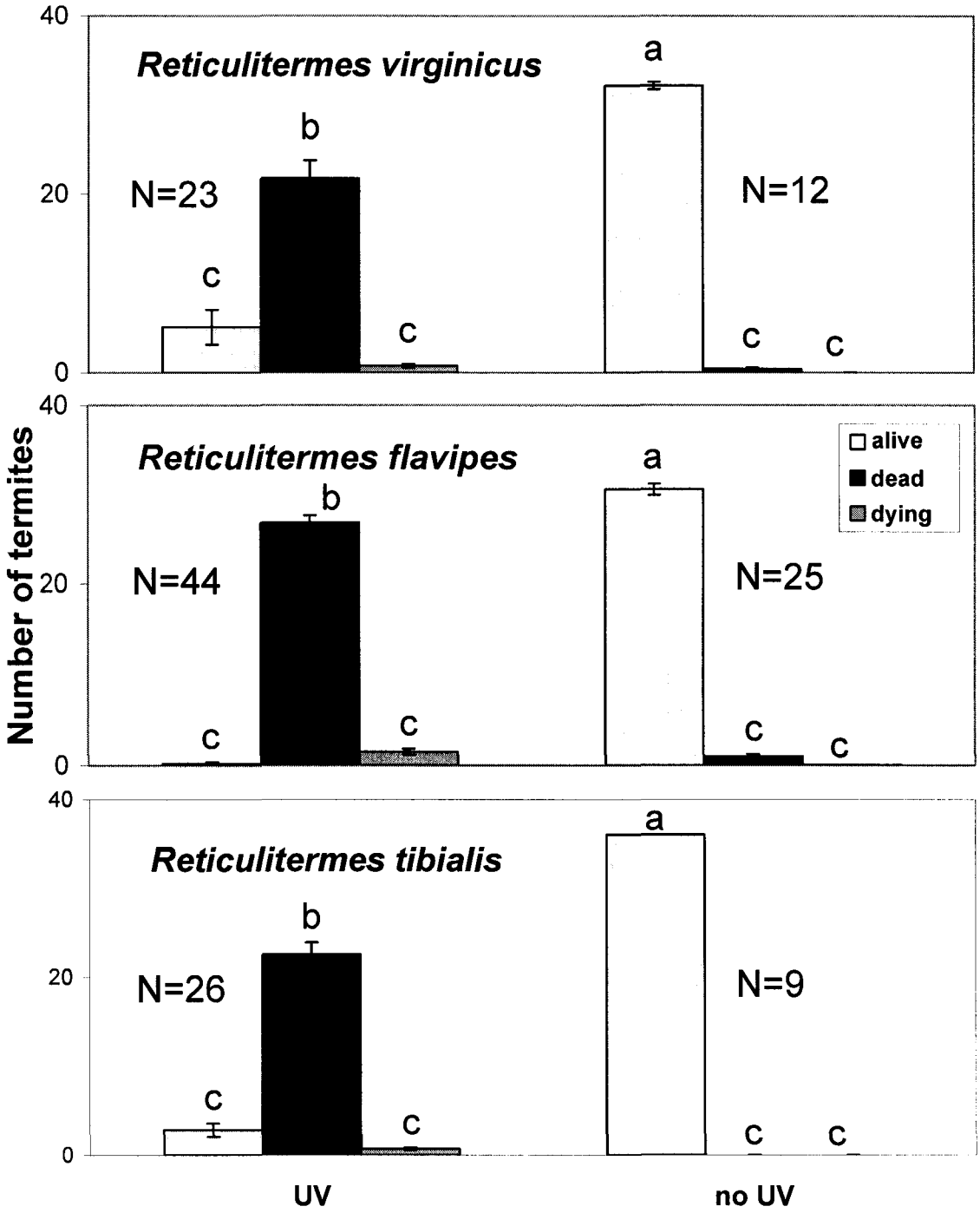

Figure 5.1 ***Reticulitermes*** **spp. UV-mediated mortality.** Mortality data was recorded after 24 hour UV light exposure (60 W/m^2). Individuals scored as 'dying' had decreased mobility and generally died within 48 hrs of treatment while termites scored as 'living' showed high mobility and remained viable for several weeks following treatment. No error bars are shown for *R. tibialis* controls because no mortality occurred. Significant differences (P<0.001, Tukey's HSD) are indicated with lower case letters.

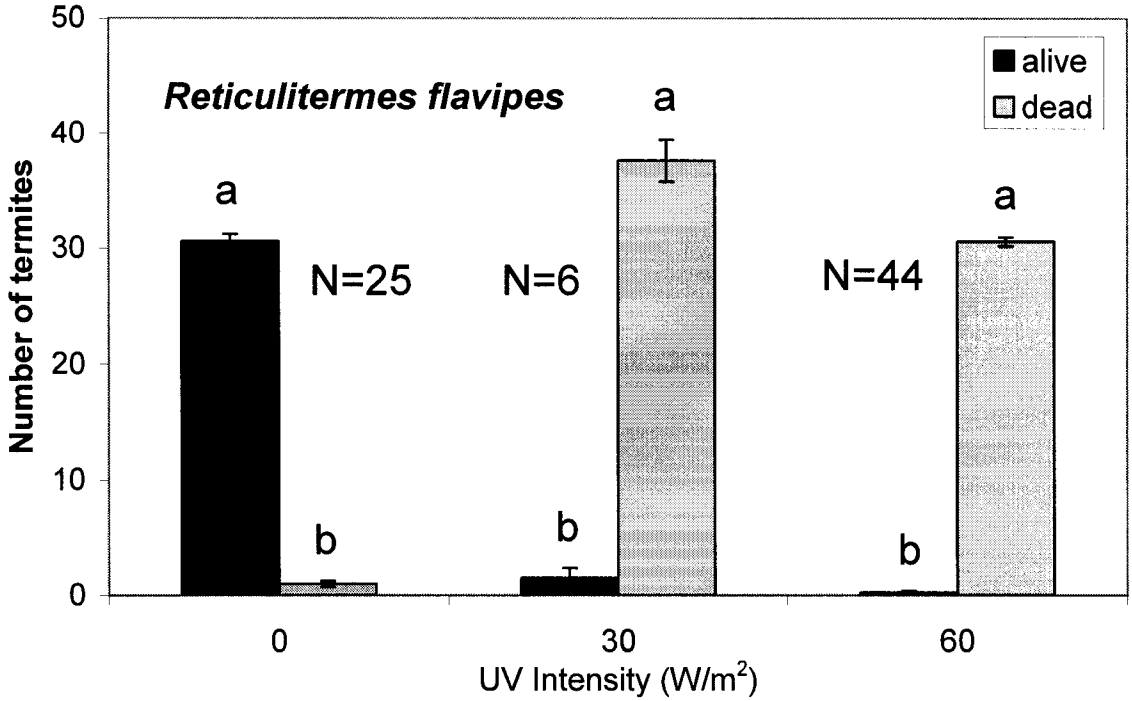

Figure 5.2 ***Reticulitermes flavipes*** **mortality at different intensities of UV light.** The UV exposure interval was 24 hours with mortality data was recorded after 48 hours. Significant differences ($P<0.001$, Tukey's HSD) are indicated with lower case letters.

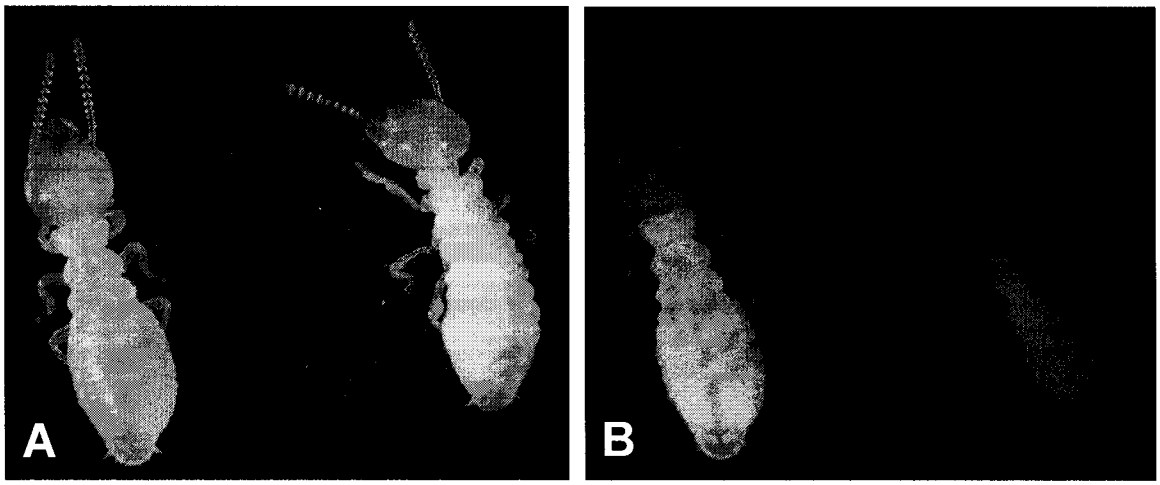

Figure 5.3 Fluorescent variation in *Reticulitermes tibialis* after UV exposure. Fluorescent variations are shown between two members of the same colony, under white (A) and ultraviolet light (B). The termite on the right in both A and B has not been previously exposed to UV light while the termite on the left has been exposed for 24 hours (60 W/m^2).

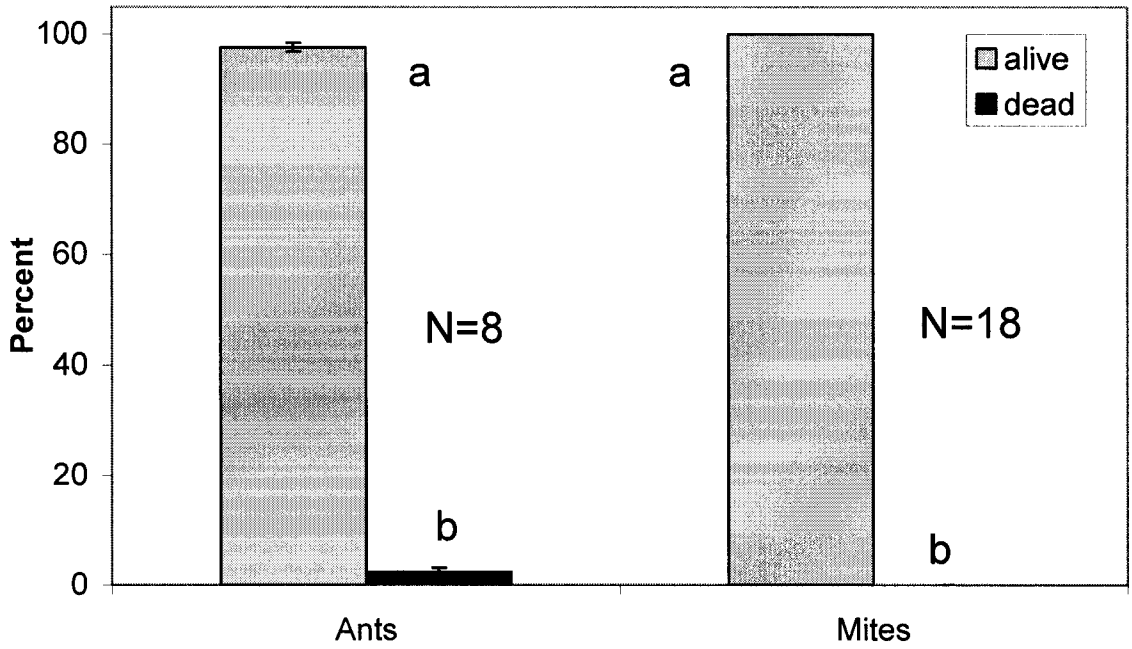

Figure 5.4 Ant and mite UV-mediated mortality. Mortality data was recorded after 24 hour UV light exposure (60 W/m^2). Significant differences (P<0.001, ants: t-test, mites: χ^2) are indicated with lower case letters. No standard error bars are shown for mites because no mortality occurred.

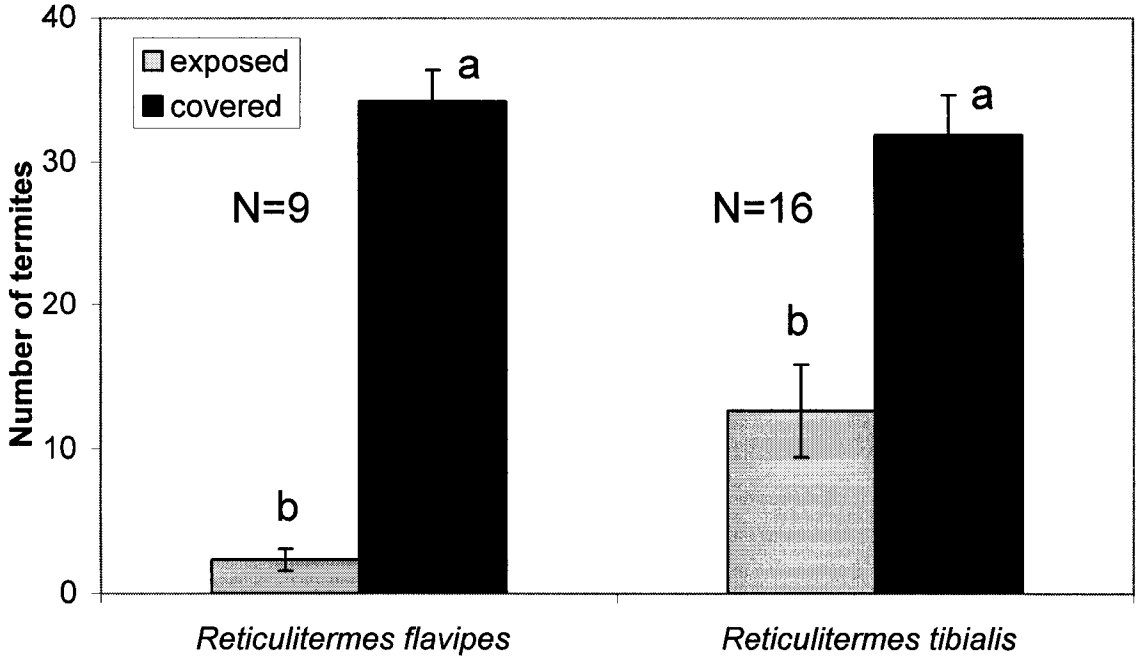

Figure 5.5 UV phototaxis bioassay data. Number of termites on the respective halves of the phototaxis bioassay arenas recorded after 24 hours UV light exposure (60 W/m^2). Significant differences (P<0.001, t-test) are indicated by different lower case letters.

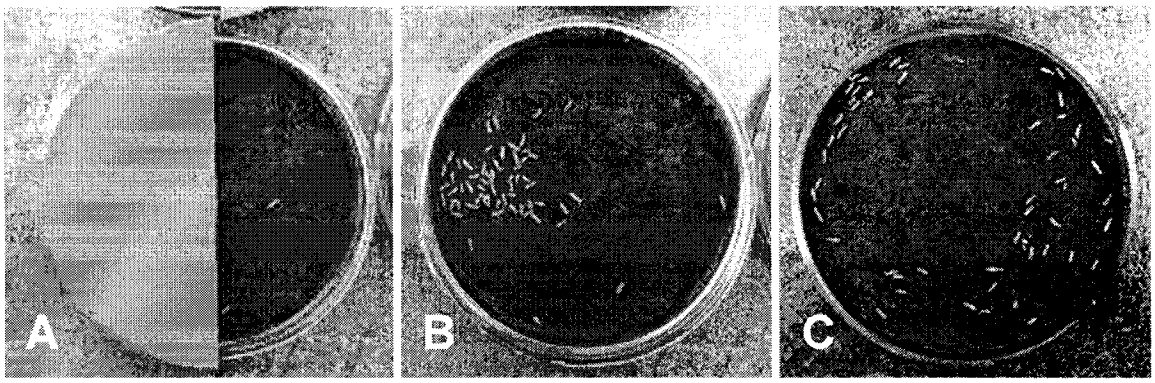

Figure 5.6 UV phototaxis bioassay. (A) Bioassay arena immediately following 24-hour UV light exposure (60 W/m^2). (B) The same bioassay arena as in A with the cardboard removed. (C) Bioassay arena showing typical termite distribution under fluorescent laboratory lighting.

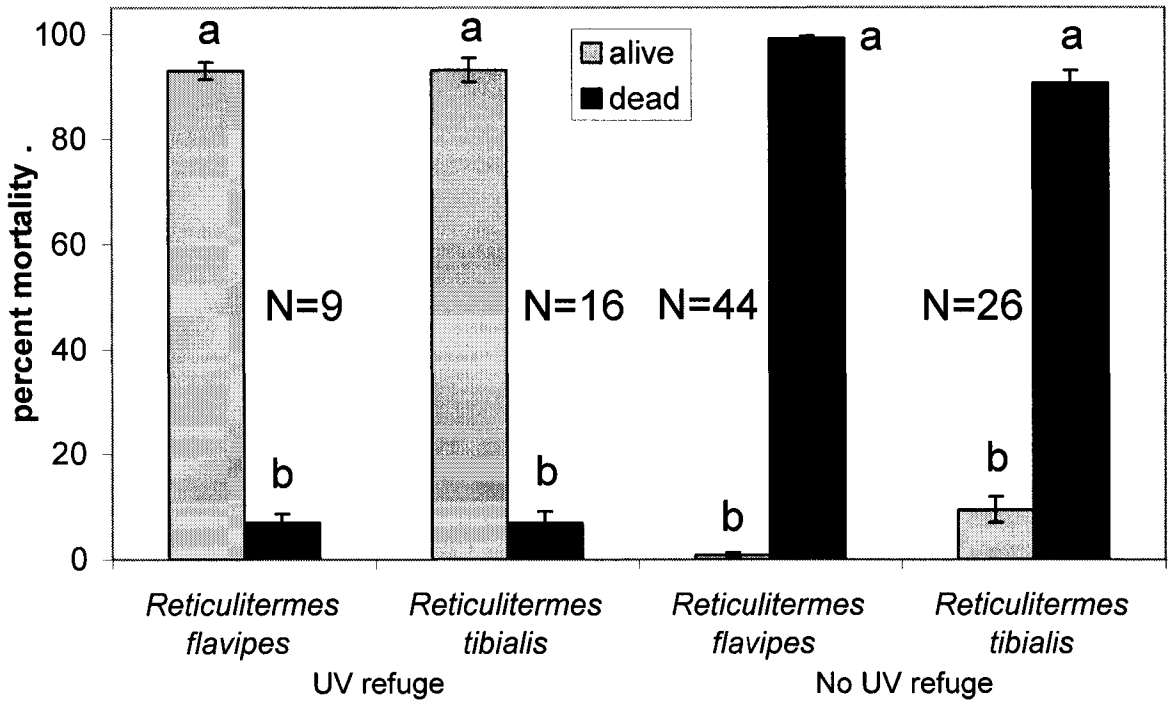

Figure 5.7 Negative phototaxis effects on UV mediated mortality. Mortality for bioassay arenas which were half-covered (UV refuge) and arenas with no cover (no UV refuge). Data was recorded after 24-hour UV light exposure (60 W/m^2). Significant differences (P<0.001, Tukey's HSD) are indicated by different lower case letters.

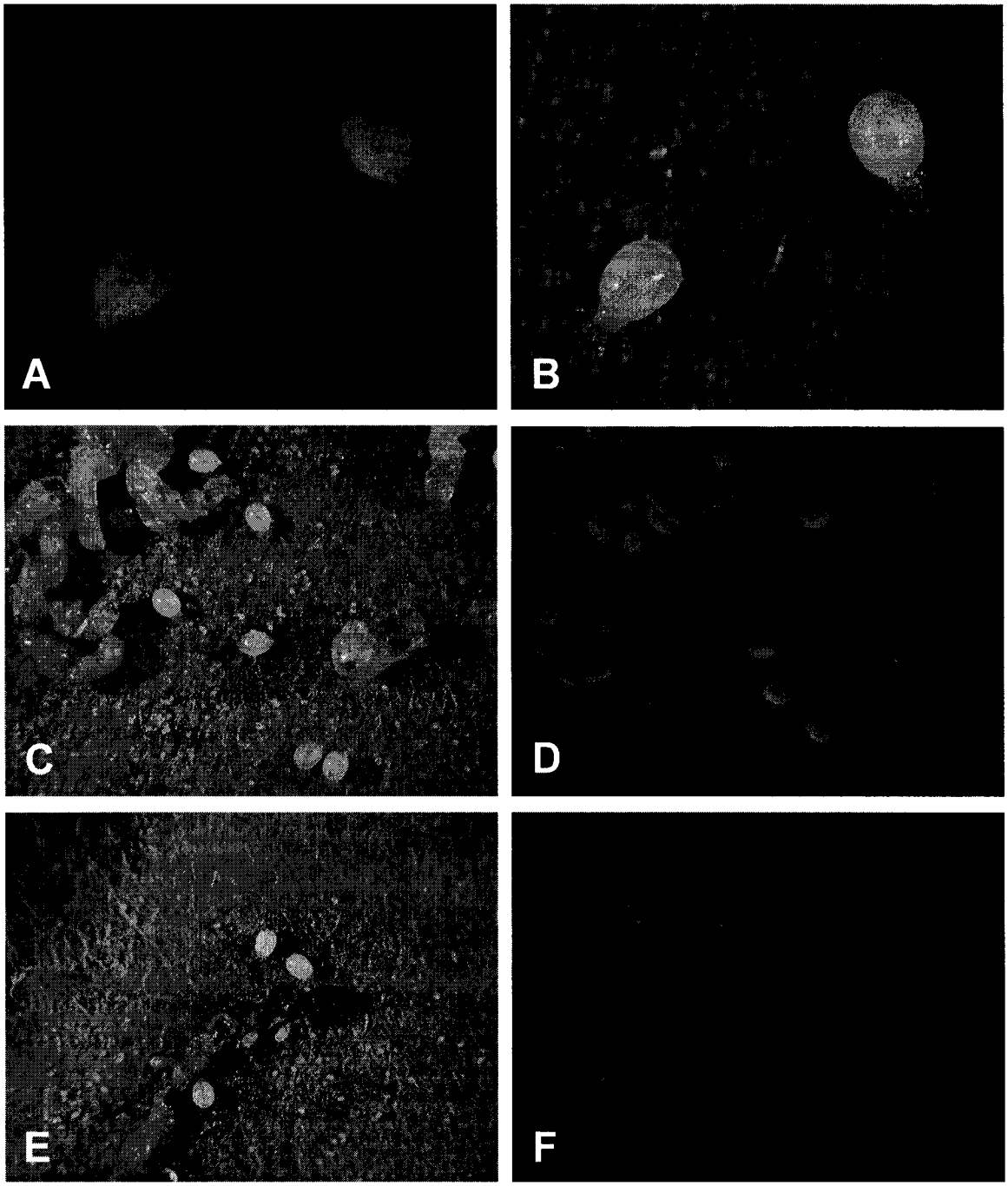

Figure 5.8 Fluorescent variation in *Tyrophagus* sp. mites fed on *Reticulitermes tibialis*. Mites fed on fluorescent *R. tibialis* carcasses, under white (A) and ultraviolet light (B). Note defined areas of more intense fluorescence under UV light (B). Fluorescence variations between mites fed on fluorescent *R. tibialis* carcasses, under white (C) and ultraviolet light (D), and mites fed on non-fluorescent *R. tibialis* carcasses, under white (E) and ultraviolet light (F).

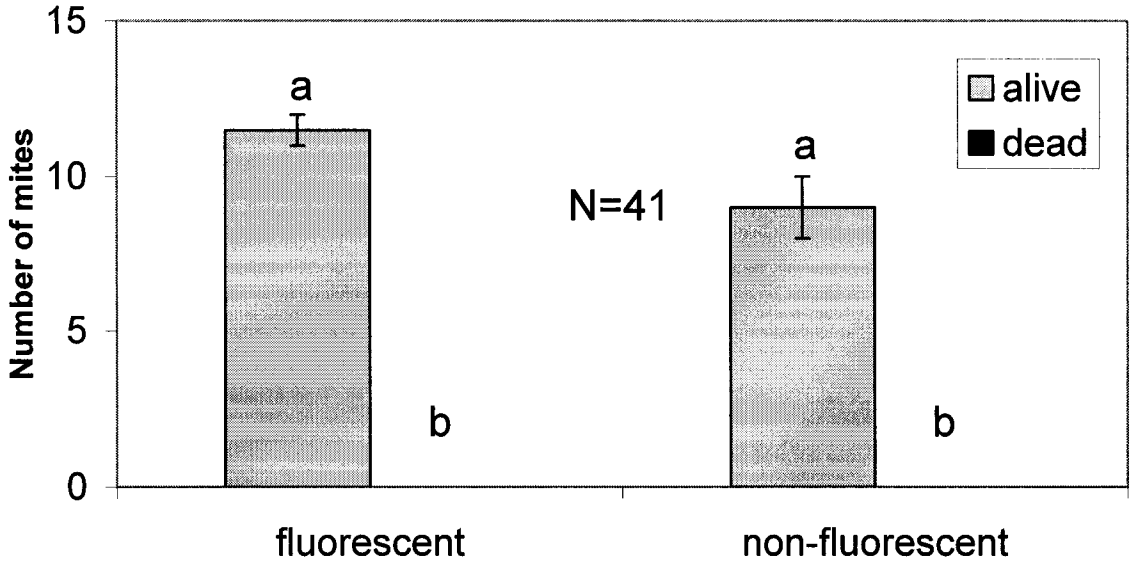

Figure 5.9 UV-mediated mortality in *Tyrophagus* sp. mites fed on *Reticulitermes tibialis*. Mortality data was recorded after 48 hour UV light exposure (60 W/m^2). Fluorescent mites, fed on fluorescent *R. tibialis,* contained norharmane. Non-fluorescent mites, fed on non-fluorescent *R. tibialis,* did not contain norharmane. Significant differences (P<0.001, χ^2) are indicated by different lower case letters.

exposure (60 W/m^2) did not cause any mortality in the fluorescent mites (Fig. 5.9) in contrast to the high mortality seen in termites. However, fluorescent mites were observed to lose their fluorescence after treatment as seen in termites. HPLC analyses show that mites that previously contained norharmane and fluoresced contained no norharmane after 24 hour UV exposure.

Ants were observed to feed on both 20% sucrose and 20% sucrose/sat. norharmane solutions. Methanol extracts from ants fed norharmane fluoresced while those fed only sucrose did not. There were no significant differences in UV-induced mortality between ants fed on a diet containing norharmane and those fed on only sucrose when exposed for 48 hours at 60 W/m^2 (Fig. 5.10).

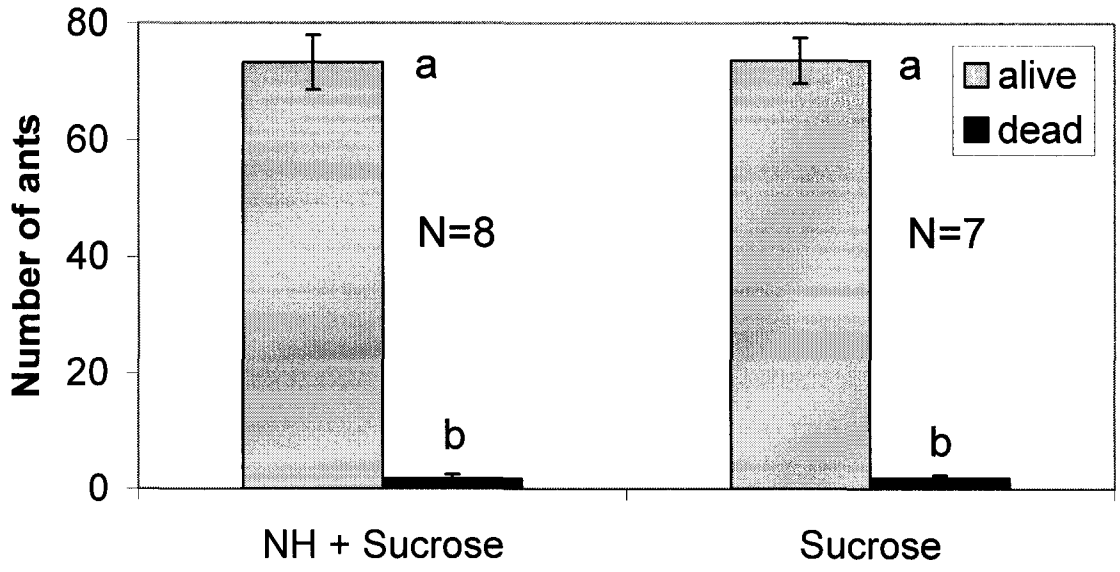

Figure 5.10 UV-mediated mortality in ants fed norharmane (NH)/sucrose or sucrose solutions. Mortality data was recorded after 24 hour UV light exposure (60 W/m^2). Significant differences ($P<0.001$, Tukey's HSD) are indicated by different lower case letters.

Discussion

Norharmane and other ß-carbolines have been studied both for their inherent toxicity and their in phototoxicity. We were therefore intrigued with the possibility that termites might be susceptible to UV-mediated phototoxic effects. *Reticulitermes tibialis, R. flavipes* and *R. virginicus* exposed to 60 W/m^2 intensity UV light for 24 hours, and *R. flavipes* exposed at 30 W/m^2, displayed high mortality, positively correlated with UV light intensity, presumably because of their high *in situ* levels of the phototoxin norharmane. This may be the first known instance of autophototoxicity in nature.

Tyrophagus mites found with the termites were also exposed to UV light, but no mortality was observed. Pavement ants were also tested with UV light, and similarly showed no mortality. Previous work with UV light and insects showed that the wavelengths and intensities used in the current experiments typically cause little or no mortality in other insect species (Guillet et al. 2000, Larson et al. 1988). The failure of UV exposure to cause mortality in the control insects in this or other studies supported the conclusion that it is the phototoxic activity of norharmane that is causing mortality in the termites and not UV light itself.

UV exposure of less intensity (15 W/m^2) for a shorter time interval (40 minutes) has previously been used to remove protozoa selectively from the termite gut (Inoue et al. 1997). This treatment caused mortality in the UV-treated termites relatively slowly (80% recorded after 20 days, comparable to starvation) compared to the more rapid mortality observed in the present study (79-94% in 24 hours). The differences to mortality between these studies demonstrate that different modes of action are involved. Selective

removal of protozoa by short term UV exposure removes a nutritional source for termites with mortality occurring presumably due to starvation. Twenty-four-hour UV exposure causes mortality caused by damage due to the reactions of the phototoxin norharmane.

We were interested to find that termites exposed to UV light lost their fluorescence within hours of UV exposure (Fig. 5.3), but control termites showed no decrease in fluorescence. HPLC analysis of UV-treated termites revealed no detectable levels of norharmane. This is consistent with a phototoxic mechanism in which norharmane is consumed through reaction with target molecules (Towers and Hudson 1987) or destroyed in a catalytic cycle (e.g. production of oxy radicals) (Larson et al. 1988).

UV-dependent insecticidal activity of phototoxic compounds was first shown with southern armyworm larvae (Berenbaum 1978, Downum 1992), and this phenomenon is now known for many insect families and classes of compounds including ß-carbolines (Arnason et al. 1992, Downum 1992). Norharmane has been shown previously to exhibit comparatively strong phototoxicity toward the generalist feeder *Trichoplusia ni* Hubner relative to other simple ß-carbolines (Larson et al. 1988). UV-phototoxicity in *T. ni* (after feeding on norharmane) and *Reticulitermes* termites (which naturally contain norharmane) suggests that norharmane may have strong toxic activity against less sclerotized insects in general. In addition to insects (Larson et al. 1988), norharmane has been shown to exhibit potent phototoxic activity against bacteria (Larson et al. 1988) and eukaryotic cells (Towers and Abramowski 1983). A series of ß-carbolines was tested against *Escherichia coli* (Migula), and norharmane had the highest relative phototoxicity based on the energy needed to inactivate the bacterium (Larson et al. 1988). Other ß-

carbolines have also exhibited phototoxicity towards viruses (Hudson et al. 1986) and fungi (McKenna and Towers 1981).

The photochemistry of norharmane has been extensively studied as it has complex interactions among its energy states in different states and solvent systems (Balon et al. 1993, Dias et al. 1992, Dias et al. 1996, Reyman et al. 1996, Reyman et al. 1997, Sakurovs and Ghiggino 1982, Varela et al. 1995b, Varela et al. 1995a, Varela et al. 2001). Norharmane shows marked changes in its absorbance and emission spectra in a pH-dependent fashion (Balon et al. 1993). This is the result of four acid-base dependent species, neutral, cationic, zwitterionic and anionic (Fig. 1). In addition, a fifth species (P) is proposed in which groups of norharmane molecules interact (Reyman et al. 1997). In nonpolar solvents only the neutral species (N*) is seen on excitation (Varela et al. 1995b). In protic solvents, such as methanol or water, two additional excited states are apparent, the cation (C*) and zwitterion (Z*) (Varela et al. 1995b) and in appropriate solvent systems P* can also be observed (Reyman et al. 1997).

SCHEME 1

$$\begin{array}{ccccc} Z^* & \leftarrow & N^* & \rightarrow & C^* \\ \downarrow & & \uparrow\downarrow & & \uparrow\downarrow \\ Z & \rightarrow & N & \rightleftarrows & C \end{array}$$

The neutral species (N) and cation (C) species can equilibrate in the ground state and excitation of the neutral and cation species is reversible by decay. All other interconversions are irreversible as indicated by the arrows. P and P* behave in a manner similar to Z and Z* (Reyman et al. 1997).

The mechanism of phototoxicity against insects for norharmane, and ß-carbolines in general, is not yet clear (Larson et al. 1988). Studies with membrane-free phages

suggest that DNA is the probable target molecule and this is consistent with the mode of action of other phototoxins (Towers and Hudson 1987). The mechanism(s) by which phototoxicity are thought to occur are either by direct interaction of the excited norharmane with DNA or through secondary production of excited state oxygen species such as singlet oxygen ($^1O_2$), superoxide (O^-_2) and/or other oxyradicals (Bloomquist et al. 1997, Larson et al. 1988, Towers and Hudson 1987). While norharmane phototoxicity against *E. coli* was shown to be oxygen dependent, the authors were not able to conclude that a relationship existed between singlet oxygen ($^1O_2$) or superoxide (O^-_2) concentration and phototoxicity (Larson et al. 1988). Experiments addressing diffusion of norharmane in a hydrophobic chromatographic medium indicated the direct binding of photoactivated neutral species norharmane to DNA (Larson et al. 1988). Intercalation with DNA is thought to cause interference with replication and transcription (Lee and Berenbaum 1993, Nii 2003). In addition, aminophenylnorharmane, which is formed from norharmane and aniline, is known to cause oxidative DNA damage (Masumura et al. 2003, Ohe et al. 2002, Ohnishi et al. 2001, Totsuka et al. 2002).

The transference of norharmane from dead termites to *Tyrophagus* sp. mites feeding on them provides an interesting opportunity to follow a toxin through trophic levels. Mites feed by sucking body fluids from their prey/host and since norharmane is known to be contained in the termite hemolymph (Siderhurst 2004b), our observation that the UV chromophore was transferred to the mites was expected. While the association of *Tyrophagus* mites with termites has not been previously reported, they are common mite genus that occupy a wide variety of niches and are well known pests of laboratory animal cultures (Krantz 1978). *Tyrophagus* belongs to the family Acaridae, which includes two

other genera of mites known to be associated with termites, *Acarus* (Myles 2002) and *Australhypopus* (Wang et al. 2002b). Mite are commonly associated with termites in field-collected material or laboratory colonies (Costa-Leonardo and Soares 1993, Myles 2002, Phillipsen and Coppel 1977a, Phillipsen and Coppel 1977b, Wang et al. 2002b), but they are among the least studied organisms associated with termites (Eickwort 1990). The nature of the relationships between mites and termites is varied. Some mites are saprophagous or phoretic (Myles 2002, Wang et al. 2002b) while others, notably in the genus *Australhypopus,* are known to cause mortality in weak termite colonies (Phillipsen and Coppel 1977a). Feeding on termite carcasses has been observed in mites associated with *Reticulitermes* spp. (Myles 2002, Wang et al. 2002b) and *Coptotermes formosanus* Shiraki (Phillipsen and Coppel 1977a, Wang et al. 2002b) but the severity of their impact on the termite colony is unclear. Myles (2002) reports interactions in which mites suppress the entomopathic fungus *M. anisopliae* by direct feeding on fungal mycelia and conidia, spreading competing bacteria and yeast and fostering quicker decomposition on termite cadavers.

Many mites are opportunistic scavengers and are physiologically resistant to toxic compounds (Mullin et al. 1982). Fat bodies play an important role in the storage and eventual excretion of toxins in insects with similar structures known in mites (Filimonova 2001) and ticks (Coons et al. 1990, Obenchai and Oliver 1973). The apparent presence of norharmane in the mite's fat bodies indicates a very different treatment of this compound than in termites. Norharmane is in the hemolymph in termites, but the restriction of norharmane to defined internal areas in mites is consistent with the treatment of toxins by other arthropods (Chapman 1998). The additional spot shown in TLC analyses of

fluorescent mites suggests that mites may also partially detoxify norharmane thorough metabolism with MFOs or other enzyme pathways (Brattsten 1992). Differences in treatment, storage and physiological distribution may account for the divergent susceptibility to UV light exposure, because termites show high mortality but mites appear unaffected or insusceptible. In termites photo-activated norharmane would be in close proximity to many biological target molecules (Siderhurst 2004b), while in mites the activated toxin may be sequestered in an area where few target molecules are available, with any damage unlikely to affect the overall health of the organism.

Similar to mites, ants that fed on aqueous solutions of norharmane also failed to show UV-mediated mortality. While *T. caespitum* are not known to be predators of termites, a related species *Tetramorium simillimum* (F. Smith) has shown predatory behavior toward *Coptotermes formosanus* Shiraki in laboratory arenas (Cornelius and Grace 1995). Both *Tetramorium* spp. are generalist ant predators and are likely opportunistic prey on termites in nature. Unlike the mites, whose cuticles are largely transparent in the UV, the ant cuticle is heavily sclerotized, blocking shorter wavelengths of light (Felisberti and Ventura 1996). This difference in cuticle composition and UV transparency may explain the insusceptibility of ants to UV-mediated phototoxicity from norharmane. The failure of norharmane to cause significant UV-mediated mortality in either ants or mites suggests that norharmane does not function as an effective defense against invertebrate predators or parasites. Phototoxins are also known to have negative effects on vertebrates (Arnason et al. 1992, Berenbaum 1991, Bourke 2000, Bourke 2003), although most references are to herbivores rather than predators. It is possible that norharmane may play some role in defense against vertebrate predators that are exposed

to substantial amounts of UV radiation. However, norharmane's primary biological role in termites is most likely as an antimicrobial defense against the range of microorganisms termites encounter and not a predation deterrent.

Laboratory observation of termites under UV light exposure led to the hypothesis that *Reticulitermes* spp. termites may be negatively phototactic with respect to UV light and actively avoid prolonged exposure. This was confirmed experimentally with termites exposed to UV light for 24 hours in half-covered petri dishes. Termites showed a strong preference for the covered half of the dish. Termite mortality was considerably reduced in these tests, demonstrating that avoiding exposure to UV light may be an effective strategy against autophototoxicity. The autophototoxic effects of norharmane for *Reticulitermes* termites are also likely minimized by their subterranean environment, in which they face little UV light exposure (Schober and Lohmannsroben 2000). This supports previous results showing that termite workers avoid shorter wavelength incandescent and fluorescent light, but show no aversion to longer wavelength red light (Cabrera and Rust 1996). Norharmane's absorbance spectrum shows that it does not absorb strongly above 360 nm, consistent with its white color, further indicating that red light would not present an autophototoxic risk. In contrast to workers and soldiers, termite alates are positively phototactic with respect to light, and this response is used as a collecting technique for swarming alates (Nalepa et al. 2001). Alates of several termite genera are known to contain norharmane (Siderhurst 2004a, Siderhurst 2004c), but all possess sclerotized and darkly pigmented cuticles which likely block most UV radiation.

While norharmane-mediated autophototoxicity may not be an ecologically relevant risk for *Reticulitermes spp.* and other largely subterranean termites, it may cause

phototoxic risks for termites that do spend appreciable time in sunlight, such as termites that forage above ground. Species that forage above ground to collect grasses, such as the harvester termite, *Hodotermes mossambicus* Hagen and the snouted harvester *Trinervitermes trinervoides* (Sjostedt) have more heavily sclerotized and pigmented cuticles. In addition to blocking UV light, termites that forage in sunlight could have reduced levels of norharmane.

Termite workers and soldiers may possess photosensors for ultraviolet light that allow them to avoid UV exposure. Previous observations have shown fluorescence surrounding the eyes of *Reticulitermes* workers and this may be the location of UV photosensors (Siderhurst 2004c). UV chromophores are known to be involved in insect sight (Eckert 1971, Kay 1969) and photoreceptors have been reported from within the brain itself (Fleissner and Fleissner 2003, Gao et al. 1999). There is also precedence for photoreception that involves areas of modified cuticle on the head that allow penetration of restricted frequencies of light (Hardie et al. 1981, Meyer 1977, Seifert et al. 1987).

Previous work has shown that some termites fluoresce with less intensity than others, although their norharmane levels do not appear to be lower (Siderhurst 2004c). Whole body pH tests indicated no differences in termites that displayed lower fluorescence although tissue level variations may exist (Siderhurst 2004c). Insects can regulate the pH of their internal fluids such as hemolymph and gut contents (Harrison 2001). Changes in pH are known to assist in dealing with toxins (Govenor et al. 1997, Timmermann et al. 1999). The photochemistry and phototoxicity of norharmane and other β-carbolines has been extensively studied but the issues concerning combinations of these two in biological systems have received little attention. In particular,

norharmane's absorbance and fluorescent spectra show pronounced variations with changes in pH (Varela et al. 1995b) and this may profoundly alter the phototoxicity experienced by organisms containing the phototoxin. It is possible that some termites change their internal pH, converting norharmane to a tautomer or molecular species that does not absorb UV light strongly and thereby derives protection if exposed to daylight.

Specialist insect herbivores can tolerate the presence of ß-carboline alkaloids and other phototoxins through biochemical and/or behavioral strategies (Arnason et al. 1992, Berenbaum 1995, Downum 1992). The leaf beetles, *Chrysolina hyperici* (Forster) and *C. quadrigemina* (Suffrian) feed as both larvae and adults on *Hypericum perforatum* (Clusiaceae) (St. John's wort), which contain the phototoxin hypericin (Fields et al. 1990). Adult beetles feed during the day and derive protection from their pigmented cuticle, which transmits less than 0.2% of the harmful wavelengths. *Chrysolina* spp. larvae are unsclerotized, similar to termite workers, feed at dawn when UV intensity is low (6 W/m^2) and retreat into the soil during the day when UV intensities are high (300 W/m^2). The cue for this behavior appears to be light, as in termite negative phototaxis, and not and not temperature (Fields et al. 1990). Another behavioral response to phototoxins has been observed in larvae of the European corn borer *Ostrinia nubilalis* Hubner, which roll themselves inside the leaves they feed on, preventing UV exposure and phototoxicity (Champagne et al. 1986). Elevated levels of antioxidant enzymes allow the black swallowtail larvae *Papilio polyxenes* F. to exploit a food resource containing phototoxins including ß-carbolines (Lee and Berenbaum 1993). Other insect defenses against phototoxins include metabolism and excretion and sequestering toxins, which may be the method used by mites containing norharmane (Arnason et al. 1992).

Interactions involving autotoxicity with respect to variation in environmental conditions have been reported in Birdsfoot trefoil, *Lotus corniculatus* L., and the clover *Trifolium repens* L. (Brighton and Horne 1977). In this instance, freezing temperatures rather then UV light induces instability in the defensive compounds (cyanogenic glycosides), which causes increased mortality in colder climates (Brighton and Horne 1977).

Termites are among the most economically important pests, with an impact approaching $11 billion annually in the United States (Su 2002) and $40 billion worldwide (Wiseman and Eggleton 1994). Despite increasing damage from termites, several soil termiticides have been recently withdrawn from the market due to environmental concerns. The occurrence of norharmane in termites and its strong insecticidal activity when activated by UV light suggests promising opportunities for termite pest control. UV sources may provide a behavioral deterrent to termites, which can be used to protect structures. Direct excitation of norharmane via UV light or ultrasound (Reyman et al. 1996) could provide control methods that leave no chemical residue and are highly termite-specific. Chemiluminescent methods may also allow excitation of the phototoxin through energy transfer agents, allowing the development of new, highly selective classes of termicides (Cepas et al. 1996).

Several reagent systems are currently used in chemiluminescence HPLC detection systems for ß-carbolines in general and several have been used specifically with norharmane. These include bis(2,4-dinitrophenyl)oxalate-hydrogen peroxide (Cepas et al. 1996), bis(2,4,6-trichlorophenyl)oxalate-hydrogen peroxide (Cepas et al. 1995), potassium permanganate-polyphosphoric acid (Pinotsis et al. 2000), and bis(2,4-dinitrophenyl)oxalate-imidazole (Lee et al. 2002). Unfortunately, all of these reagents

would likely be toxic to termites and other organisms without any participation of UV light. Energy transfer agents have at least 6 proposed modes of action and they encompass a wide variety of compound classes. It may be possible to pick energy transfer candidates using the Rehm-Weller equation with appropriate E_{oo} data for the potential compounds (Arce et al. 2003, Cho et al. 2003).

The effectiveness of energy transfer agents for controlling subterranean termites also hinges on the mechanism of norharmane phototoxicity. For energy transfer agents to work, norharmane must be toxic in a stoichiometric fashion, and direct interaction of activated norharmane with DNA or other biological target molecules would be required. If phototoxicity is mediated instead by excited state oxygen molecules, norharmane would be a catalyst, and termites would have to take in prohibitive amounts of reagent to cause toxicity.

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