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## Searching for the Soldier Pheromone: Behavioral Effects of Soldiers and Soldier-Produced Chemicals in the Formosan Subterranean Termite, *Coptotermes formosanus* (Blattodea: Rhinotermitidae))

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**SEARCHING FOR THE SOLDIER PHEROMONE:  
BEHAVIORAL EFFECTS OF SOLDIERS AND SOLDIER-  
PRODUCED CHEMICALS IN THE FORMOSAN  
SUBTERRANEAN TERMITE, *COPTOTERMES*  
*FORMOSANUS* (BLATTODEA: RHINOTERMITIDAE)**

A Thesis

Submitted to the Graduate Faculty  
of the Louisiana State University  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

in

The Department of Entomology

by  
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## Abstract

The Formosan subterranean termite, *Coptotermes formosanus*, is a eusocial insect and an invasive urban pest. Colonies function through a division of labor among castes (*i.e.*, queens and kings, workers, and soldiers). Though soldiers are energetically expensive to maintain, *C. formosanus* has a relatively large soldier caste (~10% of the colony). Soldiers are physically unable to collect food or build tunnels but are regularly found with foraging workers. In some species, soldiers influence the worker foraging behavior. In *C. formosanus*, aside from defense, the roles soldiers play remains unknown. In this study, we determined if soldiers influence the exploratory foraging of workers in *C. formosanus*. Groups of workers were introduced to planar foraging arenas to forage with different soldier concentrations. There was no significant difference in tunnel length, tunnel complexity, or food consumption within four days between foraging groups with 100 workers and 0, 2, 10, or 30 soldiers. Based on this study, no relationship was found between worker exploratory foraging activity and soldier presence. *C. formosanus* soldiers, like some other termite species, produce a defensive secretion for chemical defense. *C. formosanus* defensive secretion composition was previously investigated, showing lignoceric acid and hexacosanoic acid as two major components, but no behavioral influence was investigated. Choice assays showed *C. formosanus* workers were repelled by lignoceric acid, hexacosanoic acid, a mixture of lignoceric and hexacosanoic acids, and the crude extract of the soldier defensive secretion. Workers consumed less paper treated with the mix of lignoceric and hexacosanoic acid, indicating that the soldier defensive secretion may elicit repellant behavior in workers, allowing workers to escape from a potential threat. We additionally analyzed cuticular

hydrocarbons (CHCs) from workers and soldiers to examine differences that may identify a soldier- or worker-specific compound. No qualitative difference was detected in CHC profiles between workers and soldiers, but of the 15 peaks identified, 13 differed significantly in relative abundance. These differences may enable colony members to distinguish between workers and soldiers, but further behavioral tests are required. By investigating the multifunctional roles of soldiers in termite colonies, this study helps better understand the complex nature of communication in social insects.

## Chapter 1. Introduction

### 1.1. Formosan subterranean termites

The Formosan subterranean termite, *Coptotermes formosanus* (Shiraki), is a destructive species of the subterranean termite family Rhinotermitidae and is invasive in many subtropical climates around the world (Su & Tamashiro, 1987). They nest underground or within large pieces of wood, and a single colony can support several million individuals (Su & Scheffrahn 1988). The average life expectancy of a *C. formosanus* colony is unknown, though some field colonies have been estimated to be over 30 years old in their final stages (Grace et al. 1995).

The genus *Coptotermes* represents 18 of the 80 listed serious termite pest species. Of these, the Formosan subterranean termite is one of the most destructive (Edwards & Mill 1986; Rust & Su 2012), making it a species of great interest. It is believed to have originated from eastern mainland China and Taiwan (Kister 1985; Maruyama & Iwata 2002; Li et al. 2009), and then spread to North America, Brazil, South Africa, Pakistan, Japan, and Sri Lanka (Su & Tamashiro 1987; Evans et al. 2013).

Formosan subterranean termites were introduced to the United States through multiple introduction events, starting with Hawaii in the late 1800's (Blumenfeld et al. 2021). They have spread from initial introductions in Florida, Louisiana, and South Carolina (Chambers et al. 1988; Blumenfeld et al. 2021) to states including Alabama, California, Georgia, North Carolina, and Tennessee (Su 2003; Evans et al. 2013). They were identified in Louisiana in 1966 (Spink 1967) and likely arrived on supply boats from Asia following WWII (Su & Tamashiro, 1987). They are most heavily concentrated in southern Louisiana around New Orleans but have spread north into the state (Messenger

et al. 2002). In Florida, both *C. formosanus* and the closely related species *C. gestroi* (Wasmann) are important pests, especially in the Miami and Tampa areas (Scheffrahn & Su 2005).

## **1.2. Sociality in termites**

Eusociality has evolved multiple times in insects (Andersson 1984), once in crustaceans (Duffy 1996) and once in mammals (Jarvis 1981). It is defined by overlap of generations, cooperative brood care, and reproductive division of labor (Wilson 1971). Termites (order Blattodea) and hymenopterans (bees, ants, and wasps) are the most common examples because of their global presence and close association with humans.

Termites, like other eusocial groups, have an established caste system that divides labor between specialized members within a colony. The reproductive caste is responsible for starting a colony and producing offspring, the worker caste performs general husbandry and forages for food, and the soldier caste protects the colony from predators and competitors. Members of each caste are morphologically specialized to perform their associated tasks.

Although behaviorally similar, termite eusociality is distinct from hymenopteran eusociality. Termites undergo hemimetabolous development while hymenopterans are holometabolous. These developmental differences contribute to the difference in social structure between the two groups. The hemimetabolous termites can begin working and contributing to the colony as immatures, while the holometabolous hymenopterans only perform work as adults. Upon reaching adulthood, hymenopterans are morphologically locked into a specific caste, whereas termites have a high degree of developmental plasticity. For subterranean termites, the first two instars are called “larvae” and can



differentiate into either workers or pre-reproductives (called nymphs). Workers may differentiate further into soldiers (depending on colony-specific caste composition and environmental changes), and nymphs will eventually become reproductives (Raina et al. 2004).

Eusocial hymenopterans queens mate once and store sperm to selectively fertilize their eggs. Fertilized diploid eggs are either female workers or alates while unfertilized eggs are haploid males (drones). Drones typically live to mate then die soon after. Termites are exclusively diploid, and termite queens cannot control gender by selective fertilization. In most species, all castes contain both male and female individuals.

### **1.3. Chemical ecology of social insects**

Social insects rely heavily on pheromones for communication. Termites use pheromones to alert others of danger (Stuart 1967; Reinhard & Clement 2001), recruit nestmates to food sources (Traniello, 1981; Runcie 1987; Casarin et al. 2008; Olugbemi, 2011), identify dead nestmates (Sun & Zhou 2013), regulate caste composition (Park & Raina 2004; Mao et al. 2005), and recognize members of different castes (Howard et al. 1982; Haverty et al. 1996; Singer, 1998). These pheromones are excreted through exocrine glands in various forms that either coat the cuticle or disperse through the air, such as hydrocarbons and their derivatives, terpenes, and small volatiles.

Insect cuticles are covered in lipid waxes, usually a mixture of cuticular hydrocarbons (CHCs), that provide protection against desiccation stress (Blomquist and Jackson 1979; Hadley 1984) as well as nestmate recognition cues (Haverty et al. 1996; Singer, 1998). Social insects, including termites, use CHCs for inter- and intracolony recognition (Howard et al. 1978; Blomquist and Jackson 1979; Howard et al. 1982; Hadley

1984; Singer 1998). Castes are distinguished by the unique proportions of CHCs that nestmates detect through antennal contact (Funaro et al. 2018).

Unlike CHCs, volatiles are usually comprised of smaller molecules that allow for communication over a distance as the pheromones diffuse through the nest, enabling interaction with several individuals at once. Important functions, including alarm response (Stuart, 1967; Reinhard & Clement, 2001) and caste differentiation (Matsuura et al. 2010) are mediated by volatiles, and some have additional benefits such as microbial defense (Mitaka et al. 2017).

Soldiers of many termite species produce a chemical defense that is excreted when defending the colony from threats. In the genus *Nasutitermes* (Dudley), soldier head morphology allows the soldiers to spray their defensive secretion at a distance when disturbed. These sprays can damage the cuticles of other insects (like attacking ants or termites) while entangling them and blocking their sensilla (Baker and Walmsley, 1981). For some species, the defensive spray also acts as an alarm pheromone by repelling workers and attracting soldiers (Eisner et al. 1976; Roisin et al. 1990). *C. formosanus* soldiers produce a viscus, milky white defensive secretion that is expelled through the a small opening over their mandibles (fontanelle) during fighting (Mill 1983).

#### **1.4 Termite soldiers**

Eusocial insects have developed mechanisms to maintain homeostasis within a nest, including the regulation of caste proportions. *Coptotermes* species maintain a relatively large soldier caste, though reports on overall soldier proportions are highly variable. Haverty (1977) reports *C. formosanus* soldier proportions from 5–60%, though the commonly agreed upon number is around 10% (Smith & Mauldin 1972; King & Spink.

1974; King & Spink 1975). Colony age, season, collection method (including trap type and location), and consideration of lab reared vs. field colonies may all contribute to variation in proportion calculations. King and Spink (1974 & 1975) showed that incipient colonies of *C. formosanus*, both lab-reared and field-monitored, maintained relatively consistent soldier proportions of 10%. Su and La Fage (1999) collected entire colonies whose soldier proportions ranged from 12–28%, similar to the range of 13–22% observed in small groups of field collected termites by Haverty (1979). Assuming the average soldier proportion in *C. formosanus* is around 10%, the soldier caste is significantly larger than other subterranean termites, such as the native *Reticulitermes* species, which maintain soldier proportions around 2% or less (Haverty, 1977).

Smaller soldier caste proportions are more common in subterranean termites as soldiers are energetically expensive, and thus an increased number of soldiers would provide a challenge for maintaining homeostasis. Figure 1.1 shows the morphological differences between workers and soldiers. Soldiers have enlarged mandibles specialized for colony defense that prevent them from performing other tasks within the colony, including feeding and grooming themselves (Henderson 1998). Workers spend much of their time feeding and grooming the soldiers (Du et al. 2016), and workers are also responsible for constructing the nest and tunnels, searching for and collecting food, performing undertaking and other husbandry behaviors, and caring for the reproductives and eggs (Bignell et al. 2011; Sun & Zhou 2013). Additionally, colonies with more soldiers consume more food, requiring an input of more energy (Fei & Henderson 2002).

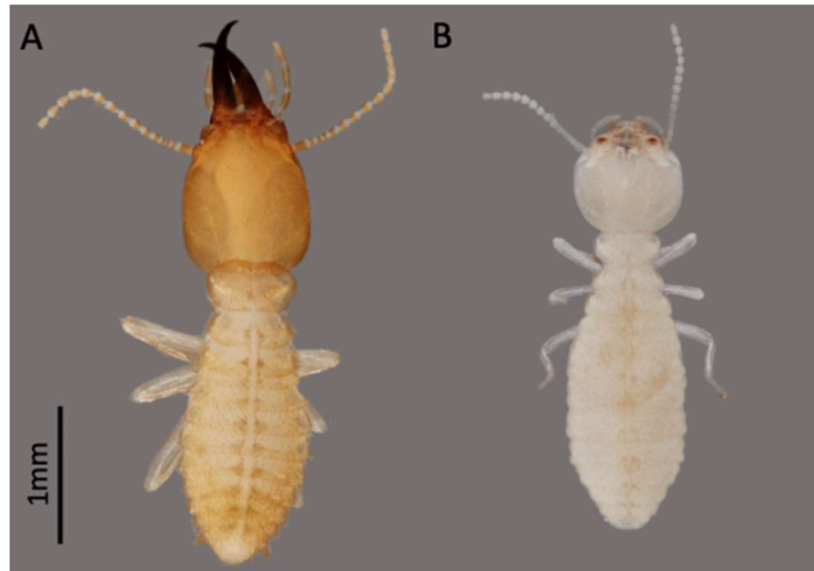


Figure 1.1. Example of *C. formosanus* worker (A) and soldier (B) morphology showing enlarged mandibles and larger, more sclerotized head capsules in soldiers than workers. Photo taken by Steven Richardson, LSU.

The main responsibility of soldiers is protection of their nest from predators (mainly ants), and the high soldier proportion in *C. formosanus* presumably evolved under predation pressure associated with a greater ant diversity in subtropical areas, but soldiers can also influence the colony in different ways. In *Reticulitermes speratus* (Kolbe), soldiers emit the volatile sesquiterpene (-)- $\beta$ -elemene, which acts as an arrestant for the workers while also exhibiting antimicrobial properties (Mitaka et al. 2017). Additionally, the presence of soldiers can affect juvenile hormone titers, which regulate differentiation from workers to soldiers (Park & Raina 2004; Mao et al. 2005), and soldier presence has been shown to influence worker tunneling behavior in three *Reticulitermes* species (Jannowiecki & Vargo 2022).

Concerning the potential multifunctional role of soldiers in *C. formosanus* colonies, the objectives of this research are to:

1. Determine if foraging behavior of workers is influenced by concentration of soldiers present;
2. Test the behavioral effects of the soldier defensive secretion and its major components;
3. Compare CHC profiles between worker and soldier castes to identify any potential differences that may be caste specific.

## **Chapter 2. Influence of soldiers on foraging behavior in the Formosan subterranean termite, *Coptotermes formosanus***

### **2.1. Introduction**

As a subterranean termite, *Coptotermes formosanus* colonies nest underground and tunnel in soil in search of cellulose-containing food. Their tunnel networks can reach over 100 meters from the main nesting site with galleries up to 50 meters long (King & Spink 1969) though their tunneling likely extends well over 100 meters in some instances. Above-ground foraging is performed under protective mud tubes constructed by the workers, often on the sides of trees or other food sources. Foraging is a risky task as termites must leave the safety of their nest to build tunnels and search for food. Termites have a variety of competitors and predators, and it is believed that ants and other species of termites are of highest concern (Cornelius & Grace, 1997). Interactions with other species are more likely when exploring new areas, and in some case may be fatal for a group of foragers, though soldiers can typically manage an attack by crowding around the entrances of the tunnels and preventing the attackers from entering (Cornelius & Grace 1997). Subterranean termites are cryptic, and there are many questions yet to be answered regarding foraging, both in Rhinotermitidae as a whole and in *C. formosanus* specifically. As one of the most significant pest species in an economically important group, answers to these questions may aid in developing better and much needed control practices.

In some species of termites, soldiers play an active role in foraging to protect foraging groups. An example of this is in the arboreal termite genus *Nasutitermes*. Termites of the species *N. costalis* (Holmgren) show specialized foraging behavior where the soldiers actively explore new areas for food as scouts. Once a food source is found,

the soldiers recruit more soldiers and eventually workers to collect and transport the food. The soldiers self-regulate their presence while providing active protection to the foraging workers (Traniello, 1981).

A study with the subterranean termite *Heterotermes tenuis* (Hagen) reported that soldiers engaged in the initiation and exploratory phases of foraging (Casarin et al. 2008), and Janowiecki and Vargo (2022) demonstrated that the presence of soldiers influenced the branching pattern and tunneling speed of *Reticulitermes flavipes* (Kollar) and tunnel construction initiation in *R. hageni* (Banks). Soldier CHC extracts were sufficient to accelerate tunnel speed in *R. flavipes*, indicating that the worker-soldier discrimination occurs at least partially through chemical communication via CHCs present on the cuticle (Janowiecki & Vargo, 2022).

The species *C. intermedius* is a significant pest in Africa and shows evidence of soldier-mediated foraging (Olugbemi 2012). They have a similar percentage of soldiers to *C. formosanus* (9–17%) with higher percentages of soldiers in foraging tunnels and galleries. The soldiers in this species typically explore new areas before the workers and, once food is discovered, they recruit the workers to collect (Olugbemi 2011). Additionally, Inita et al. (2009) showed that vibratory alarm signals produced by the soldiers of *C. acinaciformis* decreased feeding activity, suggesting that soldiers are capable of influencing food collection.

Su & La Fage (1999) showed that proportions of soldiers in *C. formosanus* are slightly larger in foraging groups, representing 14–46% of foragers, when compared to those in the nest that represent 7–23%. Soldier influence on foraging behavior may help to prevent workers from encountering dangers without soldiers present. Based on the

high presence of soldiers in *C. formosanus* and the fact that soldiers of other termite species play important roles in food exploration, we hypothesized that soldiers in this species also influence foraging behavior. To test this hypothesis, we examined the tunnel length, complexity, and food consumption of workers with the addition of different numbers of soldiers during exploratory foraging behavior.

## 2.2. Materials and Methods

### 2.2.1. Termite collection

Four *C. formosanus* colonies were collected from Bretchel Park in New Orleans, Louisiana (29°54'29"N, 90°00'32"W) using milk-crate traps filled with a lattice of 2" x 2" pine wood. Foraging groups that consisted of workers and soldiers were collected from November 2020 through May 2022 and were studied within three months of collection. Colonies were maintained in complete darkness at  $25 \pm 1^\circ\text{C}$  in clear acrylic containers (38.48 x 45.72 x 22.86 cm<sup>3</sup>) (Pioneer Plastics, North Dixon, KY, USA) with 2 cm of organic soil (Miracle-Gro All Purpose for In-Ground Use, Scotts Miracle-Gro, Marysville, OH, USA) and moistened pine wood blocks.

### 2.2.2. Foraging arena setup

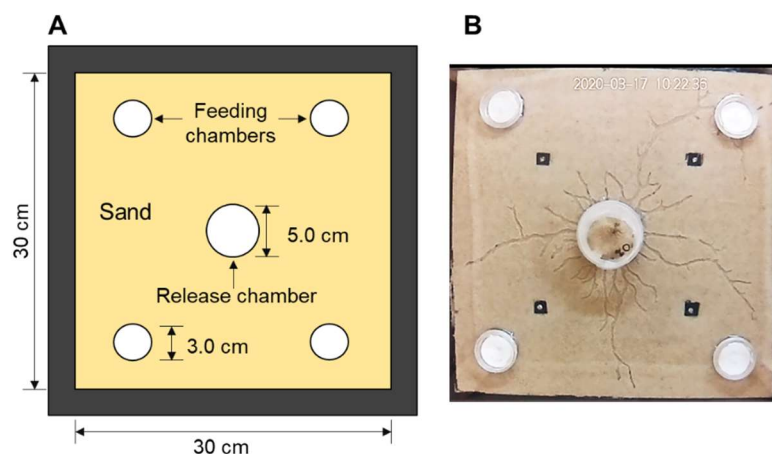


Fig. 2.1. Experimental setup. (A) Foraging arena consisting of the sand substrate, four feeding chambers, and a release chamber. (B) Representative image of foraging tunnels at the end of 96 hours.



The foraging arena (Figure 2.1) was adapted from Chouvenc et al. (2011) based on preliminary experimentation. It consisted of two Plexiglas sheets (35 x 35 x 0.6 cm) divided by a border of Plexiglas laminate (two pieces 35 x 2.5 x 0.2 cm, two pieces 30 x 2.5 x 0.2 cm), which created an open area space of 30 x 30 x 0.2 cm. Four Plexiglas laminate spacers (1 x 1 x 0.2 cm) were placed in the open area and glued to one of the Plexiglas sheets, each one ~12 cm from one of the four corners (Figure 2.1B). A hole was drilled through the Plexiglas sheets and each spacer, and a nut and bolt were used to secure the three layers, preventing the Plexiglas sheets from warping during the experiment. The top layer of Plexiglas will bore 5 holes: one for an external entrance chamber and four for external food chambers. The entrance chamber (5 cm) is in the center, and each of the food chambers (3 cm) are 10 cm from each of the four corners. All chambers were fixed to the Plexiglas sheet with hot glue before the experiment.

The arena was packed entirely with sand with 10% moisture (~1500 g). The proportion of sand to water was 10 grams of sand to 1 mL of water. The entrance chamber and each of the feeding chambers had two pre-dried filter paper discs (30 mm in diameter, Whatman grade 1, Cytiva, Marlborough, MA, USA). Each pair of filter papers received ~30  $\mu$ L of distilled water. Once in position, the Plexiglas sheets were bolted into place, and four 1-inch binder clips were applied to each side of the arena to provide additional support. A layer of hot glue was applied along the perimeter of the arena to prevent water loss during the experiment. Each of the food chambers was hot glued shut for the same reason. The entrance chamber was closed and sealed with a strip of parafilm after the termites were introduced.

### **2.2.3. Foraging behavior assay**

Four treatment groups consisting of 100 workers and either 0, 2, 10, or 30 soldiers were separated from their colony 24 hours before the start of the assay. Each group was kept in a 3 cm Petri dish with a piece of filter paper (30 mm in diameter, Whatman grade 1, Cytiva, Marlborough, MA, USA) wetted with 15  $\mu$ l of distilled water at 25°C in complete darkness. The termites were gently introduced into the entrance chamber of the arena and the lid was sealed with parafilm. Arenas were placed into their own recording chambers kept at 25°C and constant, low light levels. Termites were recorded for 96 hours using individual Raspberry Pi 3 Model B computers (Raspberry Pi, Cambridge, UK), each equipped with an Arducam OV5647 Lens Board Sensor for Raspberry Pi 3-4 with an Arducam M12 Interchangeable Lens (Arducam Technology, Kowloon, Hong Kong, CHN).

Tunnel length and intersection data were collected from video recordings at 6, 24, 48, 72, and 96 hours. Images from each time point were analyzed using ImageJ (version 1.53t). Length data were collected by measuring the pixel length of each tunnel. Pixels were converted to centimeters by using the 30 cm upper border of each arena as a reference measurement to determine pixel to centimeter ratio for each image. Intersections are defined as any point where a tunnel branched into two or more directions for more than 0.5 cm and were counted at each time point in ImageJ.

The filter paper remaining at the end of the assay was dried at 60°C for 30 minutes and weighed to measure food consumption. Mortality was examined at the end of each assay to ensure the health of the termites. Four replicates were used from each colony, and four colonies were used for a total sample size of 16 for each treatment.

#### **2.2.4. Data analysis**

Tunnel length data were analyzed using generalized linear mixed model (GLMM) with colony coded as the random effect and soldier treatment as the fixed effect to account for variation between colonies. Intersection data were compared using negative binomial regression (NB) with colony coded as the random effect and soldier treatment as the fixed effect. As many groups consumed very little to nothing, food consumption data were modeled using the zero-inflated gamma distribution with colony as the random effect and soldier treatment as the fixed effect.  $\alpha = 0.05$  was used for all tests performed. All data analyses were performed on R (version 1.4.1717) and all figures were generated using JMP Pro 16 (version 16.2.0).

#### **2.3. Results**

There were no significant differences between treatment groups at any time point in tunnel length (6 hours:  $X^2 = 2.07$ ,  $P = 0.559$ ; 24 hours:  $X^2 = 4.45$ ,  $P = 0.217$ ; 48 hours:  $X^2 = 0.83$ ,  $P = 0.842$ ; 72 hours:  $X^2 = 0.71$ ,  $P = 0.870$ ; 96 hours:  $X^2 = 1.56$ ,  $P = 0.669$ ) (Figure 2.2), intersection count (6 hours:  $X^2 = 4.18$ ,  $P = 0.243$ ; 24 hours:  $X^2 = 3.89$ ,  $P = 0.274$ ; 48 hours:  $X^2 = 1.34$ ,  $P = 0.719$ ; 72 hours:  $X^2 = 2.36$ ,  $P = 0.501$ ; 96 hours:  $X^2 = 2.05$ ,  $P = 0.561$ ) (Figure 2.3), or food consumption ( $X^2 = 1.94$ ,  $P = 0.746$ ) (GLMM,  $n = 16$ ) (Figure 2.4).

Termites performed the majority of their tunneling in the first 48–72 hours, with most groups reaching the edge of their arenas between 24 and 48 hours. Mortality was  $\leq 15\%$  in all treatments.

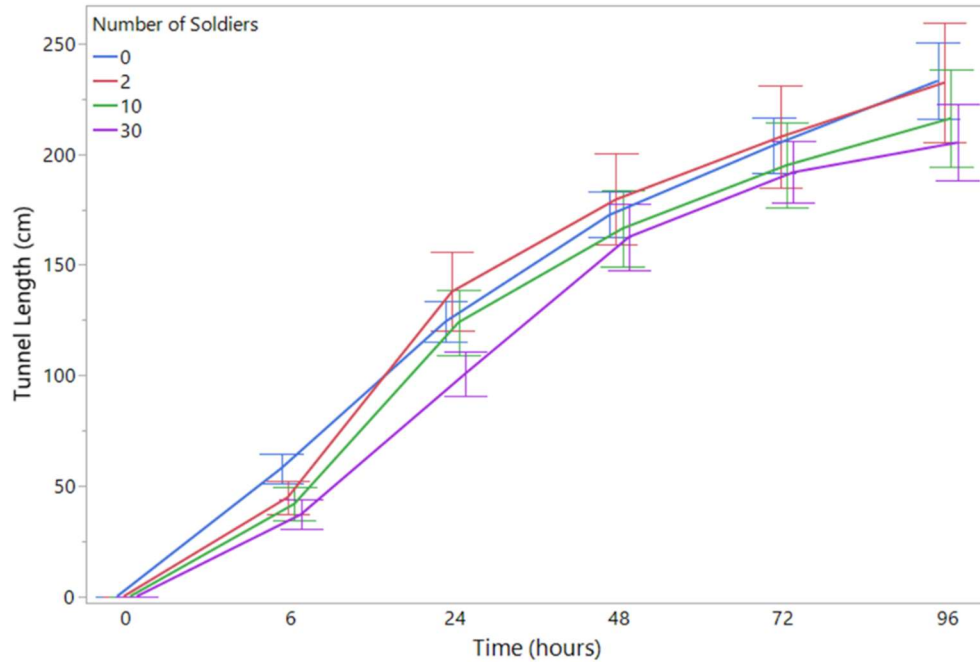


Figure 2.2. Tunnel length by groups of 100 foraging workers with different numbers of soldiers. Data shown are means  $\pm$  SEM ( $n = 16$ ).

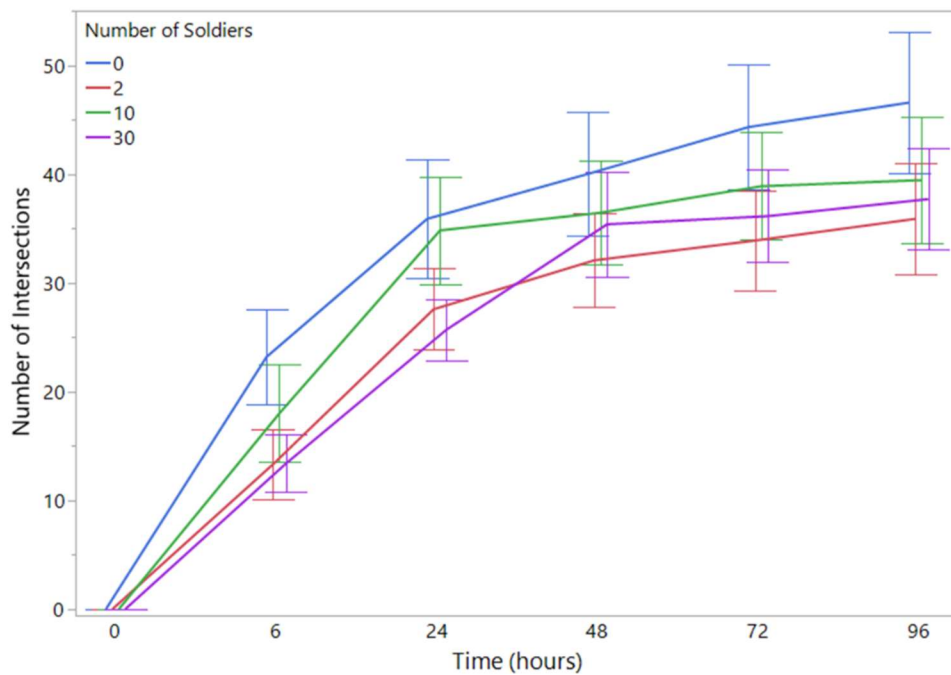


Figure 2.3. Tunnel complexity based on number of tunnel intersections constructed by groups of 100 foraging workers with different numbers of soldiers. Data shown are means  $\pm$  SEM ( $n = 16$ ).

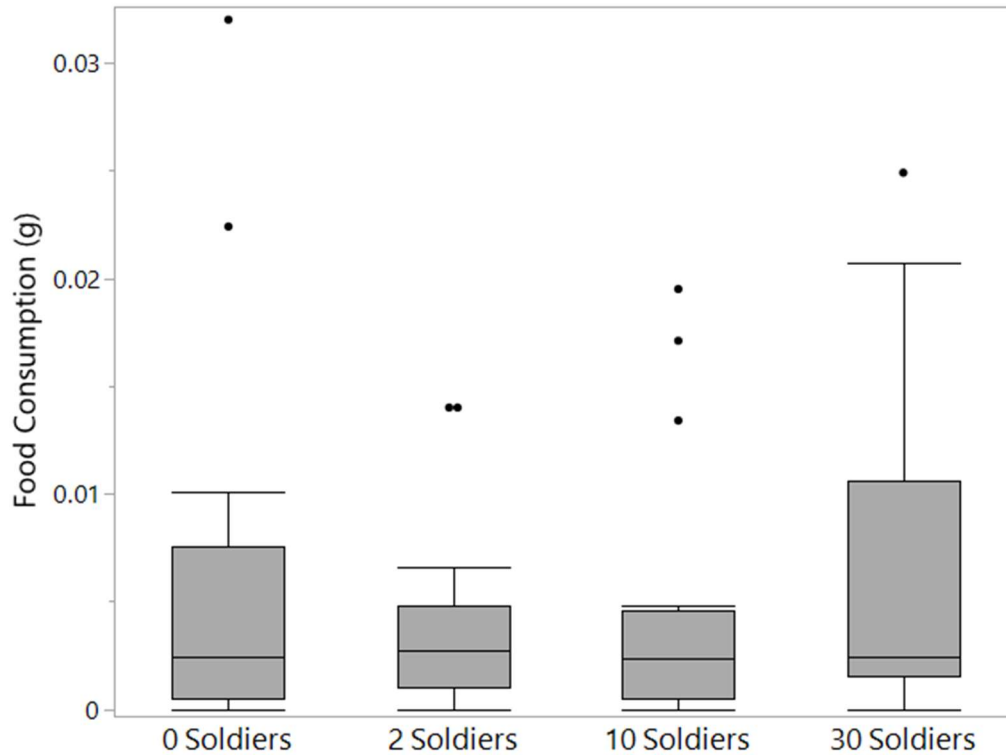


Figure 2.4. Food consumption by groups of 100 foraging workers with different numbers of soldiers. Boxes are bounded by the 25<sup>th</sup> and 75<sup>th</sup> percentiles, bands are medians, whiskers represent minimum and maximum values; and dots outside of whiskers are outliers (n = 16).

## 2.4. Discussion

In this study, no significant differences were found in foraging behavior observed between any of the treatment groups in *C. formosanus*. Soldier presence in *R. hageni* has been shown to influence the initiation of food exploration, and worker tunneling behavior was delayed by more than 10 hours in the absence of soldiers (Janowiecki & Vargo 2022). In our study, no significant difference in tunnel length was found in 6 hours between treatment groups with or without soldiers, suggesting little or no influence of soldiers on foraging initiation in *C. formosanus*.

Our results do not support the hypothesis that *C. formosanus* soldiers influence exploratory foraging behavior, but foraging workers may be influenced by soldier presence in other social contexts. Behavioral influence of soldiers may be more obvious

if foraging groups encounter predators or if their foraging galleries connect to the main nest where reproductives or brood are present. Wells and Henderson (1993) noticed that groups of *C. formosanus* workers with low soldier proportions (~2.4%) explored new chambers less regularly than groups with larger soldier proportions (~18%) in the presence of the red imported fire ant, *Solenopsis invicta*. This indicates a potential role of soldiers in foraging exploration when predators are present. Cornelius and Grace (1994) showed that *C. formosanus* worker foraging was influenced by the presence of ant semiochemicals, the presence of which slowed tunneling, but they did not explore whether soldier presence influenced this relationship. Konishi and Matsuura (2021) demonstrated that *R. speratus* workers and soldiers were more aggressive towards non-nestmate conspecifics when reproductive individuals were present rather than absent, and that workers were less aggressive in the presence of nestmate soldiers. Tian et al., (2017) also showed that *R. flavipes* soldiers alleviate worker stress in the presence of a competing termite species, *R. virginicius*. It is evolutionarily advantageous for workers to explore less with fewer soldiers, especially in the presence of enemy cues, and for workers to become more aggressive when soldiers are not present to protect them.

There is still more to understand regarding the potential roles and the mechanisms behind those roles that *C. formosanus* soldiers play within the colony. Chemical communication between soldiers and workers have been investigated in other species. For instance, the soldier-specific volatile (-)- $\beta$ -elemene acts as a worker arrestant and antimicrobial agent for *R. speratus* (Mitaka et al. 2017). Soldier presence can also affect juvenile hormone titers in *C. formosanus*, effectively regulating worker-soldier differentiation (Park & Raina 2004; Mao et al. 2005), and there are likely other important

regulatory functions the soldiers serve within the colony. Soldier pheromones and their effects await further exploration in *C. formosanus* and other closely related species of economic significance, such as *C. gestroi*.

The soldier proportion of *C. formosanus* (~10%) is relatively high compared to some subterranean termites, such as *Reticulitermes* (~2%) and *Hetermotermes* (2–5%) (Haverty 1977), though other species of *Coptotermes* maintain large soldier proportions, including *C. heimi* (~33%), *C. vastator* (~9%), and *C. intermedius* (9–17%) (Haverty 1977; Olugbemi, 2011). *C. formosanus* is native to eastern Asia (Blumenfeld et al. 2021), a subtropical part of the world that likely supports a wide variety of predators and competitors. *Reticulitermes* are native to more temperate areas, so the difference in soldier proportions may be explained by the fact that *Coptotermes* species faced more competitive and predatory evolutionary pressures that it was advantageous to spend the energy on a large soldier caste. Wells and Henderson (1993) support this hypothesis by showing that *C. formosanus* successfully fights off ant predators better than *Reticulitermes* sp. at their typical soldier concentrations (18.3% and 2.3% respectively), but not when their soldier concentration is reduced to 2.3%.

Based on the roles other termite soldiers play within their colonies, it is likely that *C. formosanus* soldiers provide more than just defensive services. Further studies focusing on potential behavioral influences of soldiers on workers (or other castes) will give us a better understanding of the complicated and cryptic nature of an economically important pest. It will also provide more context for the diversity of soldier roles within all of termites.

## **Chapter 3. Effect of the soldier defensive secretion and its major components on worker behavior in the Formosan subterranean termite, *Coptotermes formosanus***

### **3.1. Introduction**

*Coptotermes formosanus* soldiers do not rely only on their large mandibles for colony protection; like many other termite species, *C. formosanus* soldiers utilize chemical defenses alongside mechanical methods. When disturbed, a defensive secretion produced in the soldier's frontal gland is excreted through its fontanelle (Noirot, 1969; Deligne et al., 1981). While this does not produce a spray of defensive chemicals like in *Nasutitermes*, the location of the fontanelle above the mandibles allows for the defensive secretion to pool into a droplet and douse enemies as the soldiers bite them (Mill 1983). As soldiers generally guard the openings of tunnels, this location on the head is advantageous, creating a defensive barrier of snapping jaws with a sticky chemical defense. This defensive secretion is unique to the soldiers.

The frontal gland is relatively large, extending from the fontanelle into the abdomen, comprising up to 36% of the soldier's bodyweight (Deligne et al., 1981; Waller and LaFage, 1987). The defensive secretion of *Coptotermes* is a white, tacky, glue-like mixture. Compounds identified in this genus include *n*-alkanes and mucopolysaccharides in *C. lacteus* (Moore et al., 1969), hexadecanal, and heptadecanal from *C. testaceious* (Blum et al., 1982). In *C. formosanus*, the secretion is made up of free fatty acids, mucopolysaccharides, hydrocarbons, free ceramides, and other unidentified compounds (Prestwich, 1984; Ohta et al., 2007). Lignoceric and hexacosanoic acid, two fatty acids, are the major components of the secretion making up 1.7–3.4% and 0.5–0.7% of the defensive secretion, respectively (Chen et al., 1999).



Worker behavior is influenced by soldier defensive secretion in some *Nasutitermes* species by acting as a repellent (Eisner et al. 1976; Rosinj et al. 1990). It might be advantageous for *C. formosanus* workers to avoid the soldier defensive secretion because it is used during combat which workers are not adapted to. We hypothesized that the soldier defensive secretion acts as repellent for the workers, and that lignoceric and hexacosanoic acids are at least partially effective as they are the most abundant (Chen et al., 1999). To test the hypotheses, we conducted a choice assay to examine the behavior of workers exposed to two odorant sources: an untreated control and the other treated with a component or multicomponent mixture of the soldier defensive secretion.

## **3.2. Materials and Methods**

### **3.2.1. Termite collection**

Three *C. formosanus* colonies were collected from Bretchel Park in New Orleans, Louisiana (29°54'29"N, 90°00'32"W) using milk-crate traps filled with a lattice of 2" x 2" pine wood. Foraging groups that consisted of workers and soldiers were collected from April to May 2022 and were studied within four months of collection. Colonies were maintained in complete darkness at  $25 \pm 1^\circ\text{C}$  in clear acrylic containers (38.48 x 45.72 x 22.86 cm<sup>3</sup>) (Pioneer Plastics, North Dixon, KY, USA) with 2 cm of organic soil (Miracle-Gro All Purpose for In-Ground Use, Scotts Miracle-Gro, Marysville, OH, USA) and moistened pine wood blocks.

### **3.2.2. Preparation of treatments**

Authentic standards of lignoceric acid (LA) and hexacosanoic acid (HA) (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in chloroform at 1 µg/µl. Preliminary experiments showed that chloroform treated filter paper did not influence termite behavior in this assay.

The mix treatment was prepared by mixing the LA and HA solutions in their naturally occurring ratio (LA:HA = 1:3.5) in the soldier defensive secretion (SDS) (Chen et al. 1999). SDS was collected under a dissecting scope by severing the insect's head from its thorax, which forced the SDS to pool into droplets that were collected using a 10 µl capillary tube and transferred to 300 µl glass insert in a 1.5 ml glass vial (Thermo Scientific, Waltham, MA. USA). Crude extracts were suspended in chloroform and sonicated for 15 minutes before treatment to ensure the homogenization of the SDS in solution. All prepared solutions were stored at -20°C until use for experiments.

### **3.2.3. Choice assay**

The choice assay was adapted from Mitaka et al. 2017. 30 mm filter paper (Whatman grade 1, Cytiva, Marlborough, MA, USA) was cut in half and dried at 60°C for 30 minutes. After the dry mass of the filter paper was collected, all pieces were treated twice with 50 µl of either chloroform (control) or their experimental treatment (LA, HA, mix, or crude extract) with 30 minutes in the fume hood between treatments for the solvent to evaporate. Two applications were required to ensure even distribution of 100 µg of each treatment. 30 minutes after the second treatment, all pieces of filter paper were placed into their respective Petri dish and wetted with 40 µl of distilled water.

Each Petri dish had two pieces of filter paper – one treated with chloroform (control) and the other treated with one of the experimental chemicals. After groups of 20 workers were placed into the Petri dishes, they were sealed with parafilm and placed into a recording chamber with a Logitech C920 HD Pro Webcam (Lausanne, Switzerland). A total of 14 replicates were performed for each treatment using three colonies (five replicates from two colonies and four replicates from a third colony). Short recordings

(15–30 seconds) were taken at the time points of 0, 6, 24, 72, 120, and 168 hours. The recording chamber was kept in complete darkness (except when videos were recorded) at  $25 \pm 1^\circ\text{C}$ .

Termite choice count data were collected from the recordings by counting the number of termites on the control versus the treated filter paper. All 20 termites were counted at each time point, and if a termite was touching both halves of filter paper, the count was decided by which filter paper the termite had most previously been completely on. The remaining filter paper was dried at  $60^\circ\text{C}$  for 30 minutes and weighed to calculate food consumption.

#### **3.2.4. Data analysis**

Termite choice count data were analyzed using either Poisson or negative binomial regression generalized linear mixed model (GLMM) with colony coded as the random effect and treatment as the fixed effect. Food consumption data were modeled using GLMM with colony coded as the random effect and treatment as the fixed effect.  $\alpha = 0.05$  was used for all tests performed. All data analyses were performed on R (version 1.4.1717) and all figures were generated using JMP Pro 16 (version 16.2.0).

### **3.3. Results**

#### **3.3.1. Choice counts**

For the test of lignoceric acid, there were significantly more termites on the control than on the treated paper at 6 ( $X^2 = -0.45$ ,  $P = 0.003$ ), 24 ( $X^2 = -0.35$ ,  $P = 0.004$ ), 72 ( $X^2 = -0.32$ ,  $P = 0.041$ ), and 168 hours ( $X^2 = -0.30$ ,  $P = 0.012$ ) with no significant difference at 1 ( $X^2 = -0.25$ ,  $P = 0.071$ ), and 120 hours ( $X^2 = -0.14$ ,  $P = 0.338$ ) (GLMM;  $n = 14$ ) (Figure 3.1A). For hexacosanoic acid, there were significantly more termites on the control than

on the treated paper at 1 ( $X^2 = -0.46$ ,  $P = 0.002$ ), 24 ( $X^2 = -0.36$ ,  $P = 0.003$ ), and 168 hours ( $X^2 = -0.45$ ,  $P = 0.001$ ), but no significant difference at 6 ( $X^2 = 0.07$ ,  $P = 0.671$ ), 72 ( $X^2 = -0.32$ ,  $P = 0.110$ ), and 120 hours ( $X^2 = -0.18$ ,  $P = 0.265$ ) (GLMM;  $n = 14$ ) (Figure 3.1B).

For the mix of hexacosanoic and lignoceric acids, there were significantly more termites on the control than on the treated paper at every time point (1 ( $X^2 = -0.56$ ,  $P = 0.001$ ), 6 ( $X^2 = -0.53$ ,  $P \leq 0.0001$ ), 24 ( $X^2 = -0.64$ ,  $P = <0.001$ ), 72 ( $X^2 = -0.47$ ,  $P = 0.003$ ), 120 ( $X^2 = -0.50$ ,  $P = 0.024$ ), and 168 hours ( $X^2 = -0.29$ ,  $P = 0.032$ ) (GLMM;  $n = 14$ ) (Figure 3.1C).

For the crude extract, there were significantly more termites on the control than on the treated paper ( $X^2 = -0.41$ ,  $P \leq 0.001$ ) and 24 hours ( $X^2 = -0.56$ ,  $P \leq 0.0001$ ), but no significant difference was detected at any other time point (6 ( $X^2 = -0.23$ ,  $P = 0.391$ ), 72 ( $X^2 = -0.32$ ,  $P = 0.056$ ), 120 ( $X^2 = -0.24$ ,  $P = 0.154$ ), 168 ( $X^2 \leq 0.001$ ,  $P = 1$ ) (GLMM;  $n = 14$ ) (Figure 3.1D). There were no significant differences in termite counts between the two pieces of filter paper that were both treated with chloroform (method control) at any time point (1 ( $X^2 = -0.20$ ,  $P = 0.095$ ), 6 ( $X^2 = 0.13$ ,  $P = 0.321$ ), 24 ( $X^2 = 0.09$ ,  $P = 0.473$ ), 72 ( $X^2 = 0.20$ ,  $P = 0.095$ ), 120 ( $X^2 = 0.11$ ,  $P = 0.339$ ), and 168 hours ( $X^2 = -0.10$ ,  $P = 0.403$ ); GLMM;  $n = 14$ ) (Figure 3.1E).

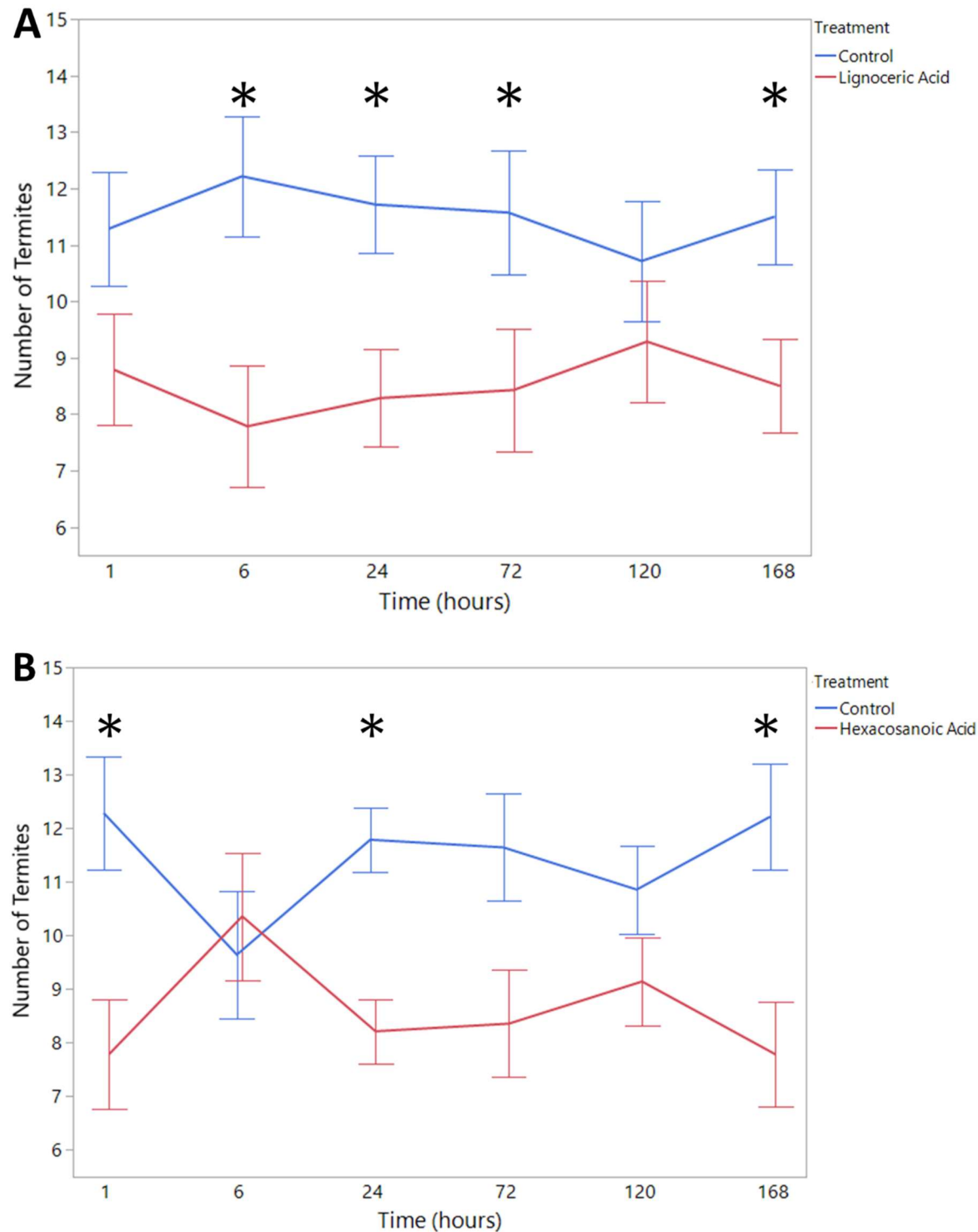
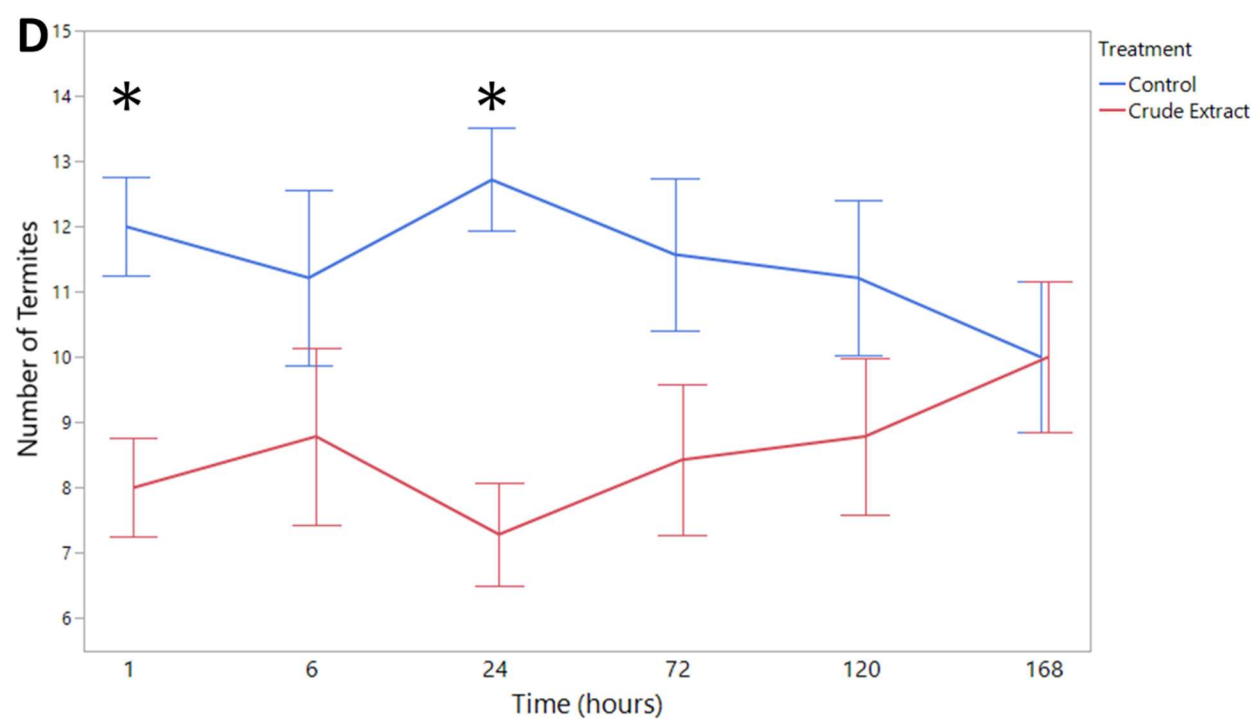
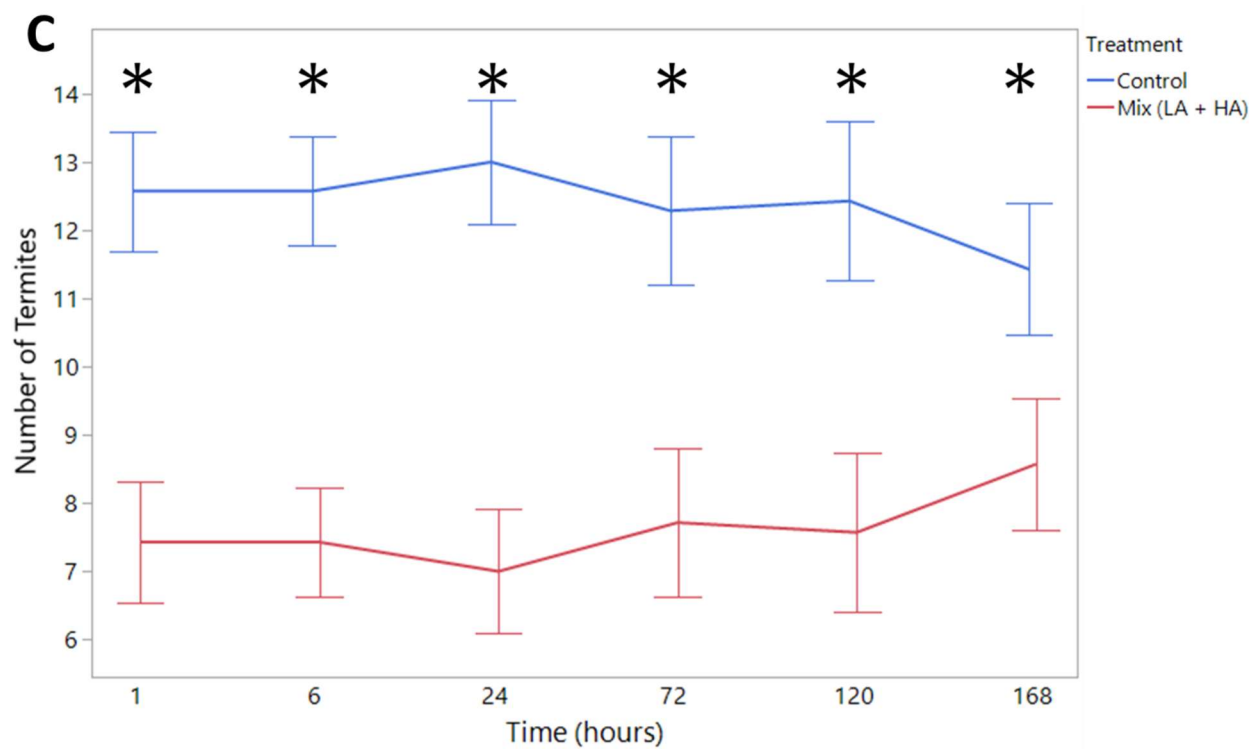
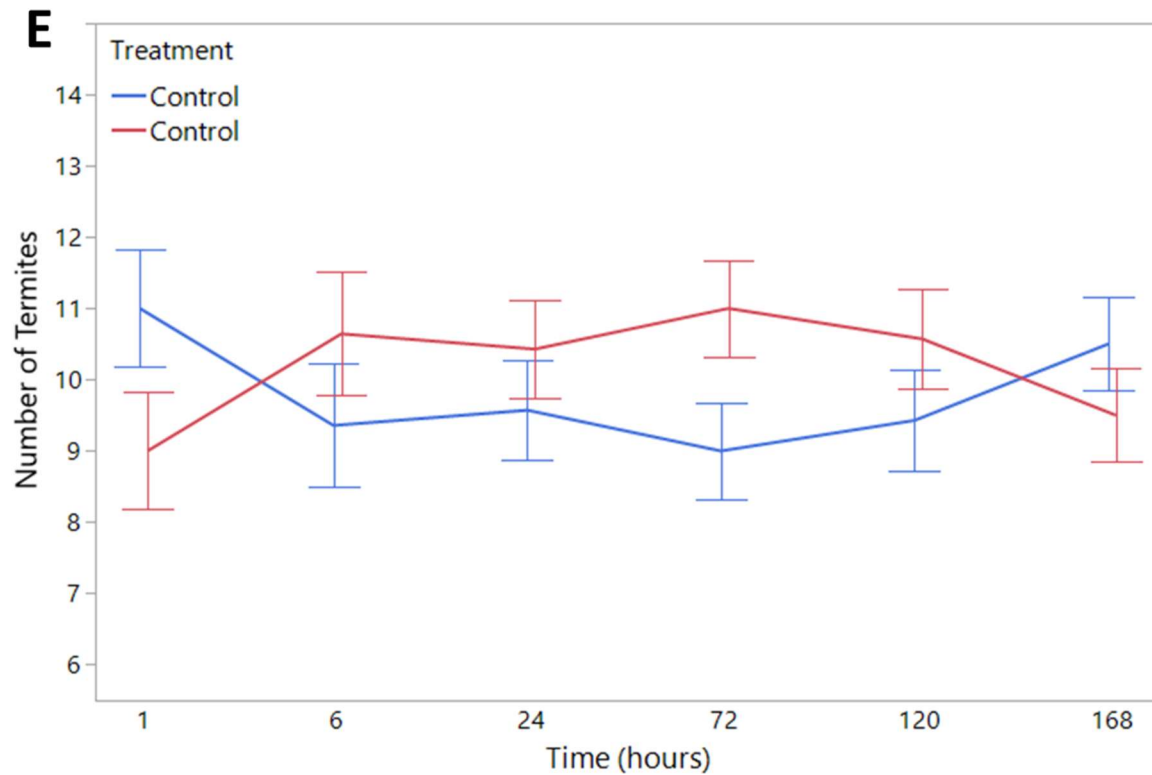


Figure 3.1. Termite choice response to filter paper treated with chloroform (control) or (A) lignoceric acid, (B) hexacosanoic acid, (C) mix of lignoceric and hexacosanoic acids, (D) crude extract, and (E) chloroform (method control). Asterisks indicate significant difference ( $P < 0.05$ , Poisson or negative binomial regression GLMM,  $n = 14$ ) at that time point. Data are shown as means  $\pm$  SEM. (Fig. cont'd)



(fig. cont'd.)



### 3.3.2. Food consumption

Termites consumed significantly less of the filter paper treated with the mix of lignoceric and hexacosanoic acids than the control paper ( $X^2 = 23.90$ ,  $P \leq 0.0001$ ), but there was no significant difference in food consumption between the control and paper treated with lignoceric acid only ( $X^2 = 2.97$ ,  $P = 0.085$ ), hexacosanoic acid only ( $X^2 = 0.34$ ,  $P = 0.559$ ), or crude extract of soldier defensive secretion ( $X^2 = 0.57$ ,  $P = 0.45$ ). There was no significant difference in food consumption between pieces of filter paper that were both treated with chloroform (method control) ( $X^2 = 0.98$ ,  $P = 0.323$ ) (GLMM;  $n = 14$ ) (Figure 3.2)

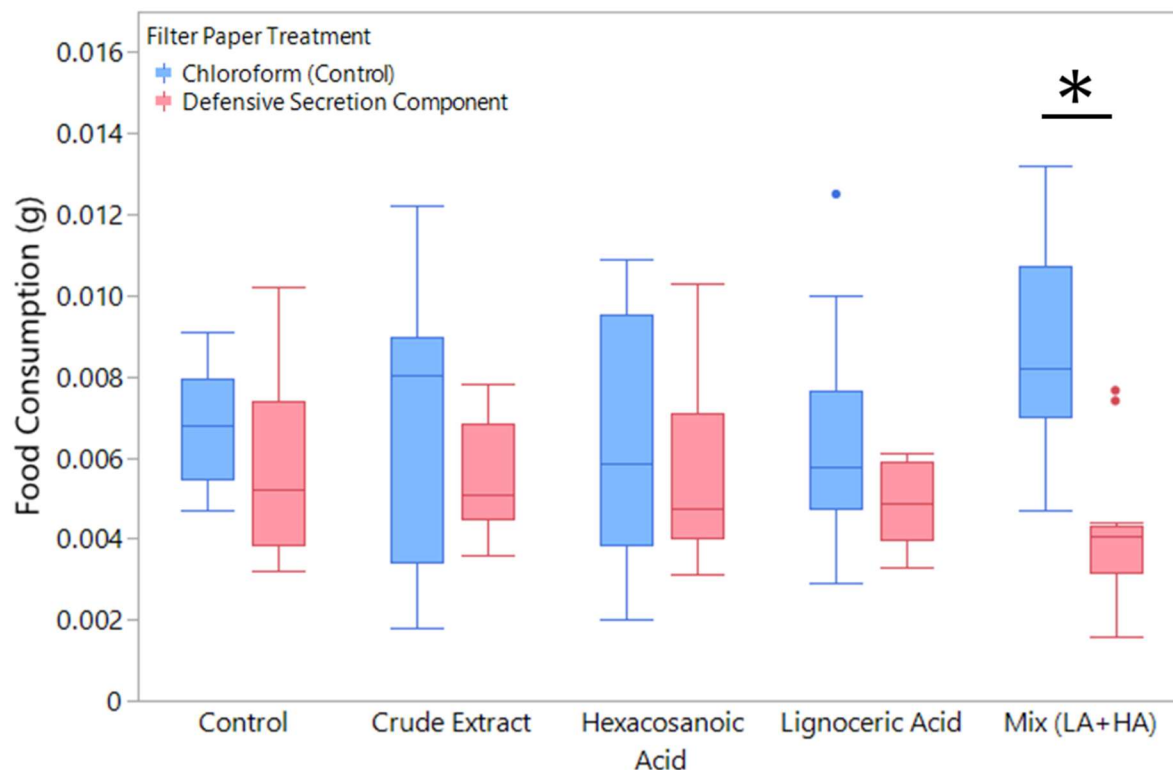


Figure 3.2. Comparison of filter paper consumption of the control and experimentally treated filter paper. Boxes are bounded by the 25<sup>th</sup> and 75<sup>th</sup> percentiles, bands are medians, whiskers represent minimum and maximum values; dots outside of whiskers are outliers and asterisk indicates significant difference in food consumption ( $P < 0.05$ , GLMM,  $n = 14$ ).

### 3.4. Discussion

Waller and La Fage (1987) describe *C. formosanus* termite soldiers as “...reservoirs of defensive fluid attached to chitinous heads” as they produce and store such large amounts of their defensive secretions. This indicates that the function of the defensive secretion is likely critical for colony defense, and as soldiers exist in such large numbers, it may play other important roles within the colony.

We report here that some of the major components of *C. formosanus* soldier defensive secretion can influence the behavior of the workers at a dose of 100  $\mu$ g. Lignoceric and hexacosanoic acid on their own acted as a repellant at some time points



(Figure 3.1A–B), but when mixed proportional to their relative quantities in the crude extract, the repellant behavior was more apparent at every time point (Figure 3.1C–D).

Workers will likely only experience soldier defensive secretions if they find themselves in the middle of an attack by a predator or competitor. They must navigate a complicated array of mechanical, audio, and chemical cues to find safety, so the repellant behavior observed by the mix of lignoceric and hexacosanoic acid may be a danger-avoidance response developed by workers. The soldier defensive secretion may act as an attractant to bring more soldiers out to defend, and this hypothesis needs to be tested in future studies.

Behavioral responses to chemical cues can be released from a single compound, but often a specific suite of chemical cues is required to elicit a full behavioral response. This may be the case with the mixture of lignoceric and hexacosanoic acids, which is further supported by the fact that workers consumed significantly less of the filter paper treated with the mixture than the control (chloroform) (Figure 3.2). There are likely other minor components of soldier defensive secretion (and other chemical cues in the form of volatiles, cuticular hydrocarbons, etc.) that influence worker behavioral response as well, so further studies can focus on behavioral response of workers to soldier defensive secretion components in the presence of some stress, such as introduction of a predator or physical disturbance. In addition, the major components should also be analyzed at different concentrations through serial dilution to determine the effective amount of each component.

In other termites, defensive secretions are responsible for a variety of physiological and behavioral responses. In *Nasutitermes*, defensive secretions can act as a repellant

for workers and an attractant for soldiers (Eisner et al., 1976) as well as having compounds that influence soldier differentiation/regulation within the colony (Lefeuve and Bordereau, 1984). Mao et al. (2005) showed that while the crude extract of the defensive secretion did not influence soldier differentiation, some of its fatty acid components stimulated soldier formation. Though there may not be a direct relationship between soldier defensive secretions and worker-soldier differentiation, these results suggest a complicated chemical and physiological relationship that has not been fully explored.

## **Chapter 4. Comparative analysis of cuticular hydrocarbon profiles of workers and soldiers of the Formosan subterranean termite, *Coptotermes formosanus***

### **4.1. Introduction**

Cuticular hydrocarbons (CHCs) form a waxy layer on the insect cuticle to provide protection from environmental stress such as desiccation, abrasion, and pathogens (Blomquist and Jackson 1979; Hadley 1984). They have evolved as semiochemicals important for both inter- and intra- species communication (Haverty et al. 1996; Singer 1998). They are especially important for social insects, including termites and eusocial hymenopterans, caste recognition is crucial for effective division of labor and colony success (Greene et al. 2003).

CHCs are used by some social species to recognize other species (Bagnères et al. 1991) and caste members in termites (Blattodea) and hymenopterans (Howard and Blomquist 1982, Sledge et al. 2001; Abdalla et al. 2003; Martin 2009, Van Oysraeyen 2014; Funaro et al., 2018). There are several examples in termites of caste-based differences in CHC profiles, including queen- and king-specific CHCs in *R. flavipes* (Funaro et al. 2018), *Cryptotermes secundus* (Weil et al. 2009), and differences in CHCs between workers and soldiers in *R. flavipes* (Darrrouzet et al. 2014).

*C. formosanus* CHC profiles have been analyzed previously and are composed of *n*-alkanes and methyl-branched alkanes (Haverty et al. 1996). Profiles of CHCs are known to fluctuate within a colony based several factors including seasonal changes, age of the colony, geographical location, and lab rearing conditions (Prestwich 1985; Haverty et al. 1990; Haverty et al. 1996; Florane et al. 2004; Perdereau et al. 2010). Haverty et al (1996) reported a quantitative difference between CHC profiles of *C. formosanus*

workers and soldiers from Hawaii populations. Such differences remain to be examined in other geographic populations, and the behavioral influence of CHCs on caste recognition is unknown for *C. formosanus*.

For a colony to operate successfully, workers, aside from their other tasks, must care for the other caste members. Workers must be able to identify soldiers within the colony, presumably through soldier-specific chemicals other castes do not produce, possibly through differences in CHC profile. We hypothesized that *C. formosanus* workers and soldiers collected from Louisiana have different CHC profiles. To test this hypothesis, we collected the CHCs of worker and soldier termites from New Orleans, Louisiana, identified the compounds using gas chromatography-mass spectrometry (GC-MS), and analyzed the differences between the two castes.

## **4.2. Materials and Methods**

### **4.2.1. Termite collection**

Three *C. formosanus* colonies were collected from Bretchel Park in New Orleans, Louisiana (29°54'29"N, 90°00'32"W) using milk crate traps filled with a lattice of 2' x 2" pine wood. Colonies were collected in November 2021 and were used within one week of collection. Colonies were maintained in complete darkness at  $25 \pm 1^\circ\text{C}$  in clear acrylic containers (38.48 x 45.72 x 22.86 cm<sup>3</sup>) (Pioneer plastics, North Dixon, KY, USA) with 2 cm of organic soil (Miracle-Gro All Purpose for In-Ground Use, ScottsMiracle-Gro, Marysville, OH, USA) and moistened pine wood blocks. Colonies consisted of workers and soldiers at time of collection.

### **4.2.2. CHC collection and analysis**

One hundred soldiers and 100 workers were used from each of the three colonies. Collections from each colony consisted of 10 groups of 10 workers and 10 groups of 10 soldiers. Each group was freeze-killed for 15 minutes at -20°C then placed into a 2 ml glass vial (Thermo Scientific, Waltham, MA, USA). Termites were washed with 100 µl of hexane with *n*-C19 at 10 ng/µl (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard for 10 minutes with gentle mixing throughout. The wash was transferred to a 300 µl glass insert in a 1.5 ml vial. The CHC samples were stored at -20°C before analysis using gas chromatography–mass spectrometry (GC-MS) (GC: Trace 1310 GC in splitless mode; MS: ISQ 7000; column: TG-5MS, 30 m × 0.25 mm × 0.25 µm; Thermo Scientific, Waltham, MA). Helium was used as the carrier gas (1.0 ml/min). Column temperature was programmed to increase from 90°C to 150°C at 20°C/min, and then to 320°C at 10°C/min with 5 min hold at 320°C. Injection temperature was 280°C, MS source temperature was 310°C, and transfer line was 300°C. The MS was set to scan a mass range from 45 to 550. Standard mixture of linear alkanes (C7 to C40) (MilliporeSigma, Burlington, MA) were injected following the same temperature program. CHCs were tentatively identified based on electron ionization mass spectra, Kovats indices, and previously published data in *C. formosanus* (Haverty et al. 1990; Haverty et al 1996). Fifteen CHC peaks were analyzed, excluding peaks that were <1% of total CHCs in both worker and soldier samples. Each peak was normalized to total CHCs in the corresponding sample based on peak area.

#### **4.2.3. Data analysis**

Comparison of relative percentage of each peak between workers and soldiers was conducted using generalized linear mixed model (GLMM) with colony coded as a random

effect and caste as the fixed effect. The principal component analysis (PCA) of the 15 peaks was conducted to compare work and soldier CHC profiles.  $\alpha = 0.05$  was used for all tests performed. All data analyses were performed in R (version 1.4.1717) and all figures were generated using JMP Pro 16 (version 16.2.0).

### 4.3. Results

A total of 15 peaks were analyzed and identified for workers and soldiers of *C. formosanus* (Table 4.1, Figures 4.1 and 4.3). No alkenes other cuticular lipids were detected, though the freeze-killed soldiers may have released a small amount of defensive secretion during the wash process causing minor contaminant peaks that were <1% of the total area and ignored for this study.

Of the 15 peaks, 13 were significantly different in relative proportion between workers and soldiers. Five were more significantly abundant in workers, including peak 1 (P1) (2-meC25) ( $X^2 = 15.50$ ,  $P \leq 0.0001$ ), P2 (3-meC25) ( $X^2 = 19.3$ ,  $P \leq 0.003$ ), P4 (2-meC26) ( $X^2 = 1.60$ ,  $P \leq 0.0001$ ), P7 (2-meC27 + 9,13-dimeC27) ( $X^2 = 12.53$ ,  $P \leq 0.001$ ), and P8 (3-meC27) ( $X^2 = 10.127$ ,  $P = 0.0001$ ) (GLMM;  $n = 30$ ). Eight of the 15 peaks were significantly more abundant in soldiers including P3 (*n*-C26) ( $X^2 = 395.6$ ,  $P \leq 0.0001$ ), P5 (*n*-C27) ( $X^2 = 108$ ,  $P \leq 0.0001$ ), P6 (9-; 11-; 13-meC27) ( $X^2 = 7.45$ ,  $P = 0.006$ ), P9 (*n*-C28) ( $X^2 = 15.16$ ,  $P \leq 0.0001$ ), P10 (11-; 13-; 15-meC28) ( $X^2 = 30.21$ ,  $P \leq 0.0001$ ), P11 (*n*-C29) ( $X^2 = 75.20$ ,  $P \leq 0.0001$ ), P12 (9-; 11-; 13-; 15-meC29) ( $X^2 = 63.49$ ,  $P = <0.0001$ ), and P15 (3-meC30) ( $X^2 = 4.096$ ,  $P = 0.043$ ). The remaining two peaks that did not differ significantly between workers and soldiers include P13 (13,15-dimeC29) ( $X^2 = 3.59$ ,  $P = 0.058$ ) and P14 (2-meC30) ( $X^2 = 0.026$ ,  $P = 0.873$ ) (GLMM,  $n = 30$ ). (Table 4.1, Figures

4.1 and 4.3). PCA of these CHCs showed a strong separation between castes (Figure 4.2).

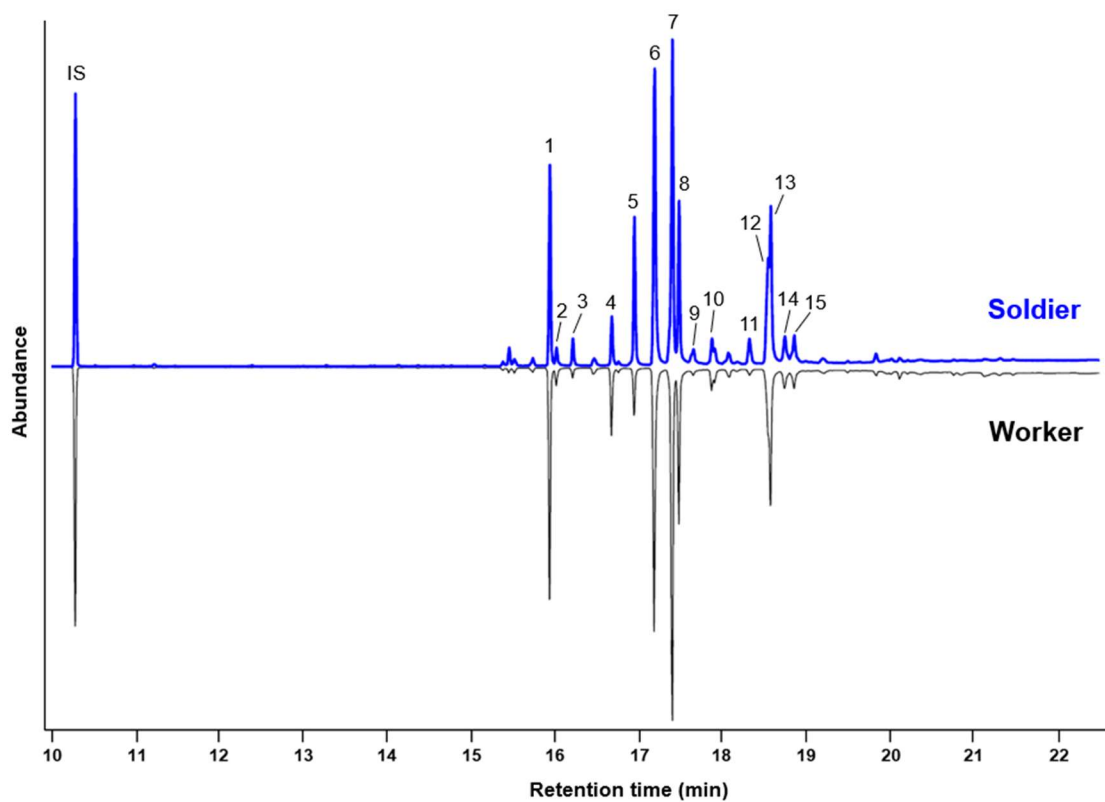


Figure 4.1. Chromatogram of CHCs in *C. formosanus* soldiers (top) and workers (bottom). IS: Internal standard (*n*-C19). Peak numbers correspond to peaks in Figure 4.3 and Table 4.1.

Table 4.1. Identification of CHCs in *C. formosanus*. Peak numbers correspond to peaks in Figures 4.1 and 4.3.

Peak no.	Identity	Retention time (min)	Kovats index
1	2-meC25	15.932	2564
2	3-meC25	16.013	2574
3	<i>n</i> -C26	16.207	2600
4	2-meC26	16.673	2664
5	<i>n</i> -C27	16.942	2700
6	9-; 11-; 13-meC27	17.180	2734
7	2-meC27 + 9,13-dimeC27	17.394	2764
8	3-meC27	17.473	2775
9	<i>n</i> -C28	17.646	2800
10	11-; 13-; 15-meC28	17.867	2832
11	<i>n</i> -C29	18.313	2900
12	9-; 11-; 13-; 15-meC29	18.537	2931
13	13,15-dimeC29	18.568	2936
14	2-meC30	18.768	2966
15	3-meC30	18.850	2979

#### 4.3.1. Comparison of CHC abundance

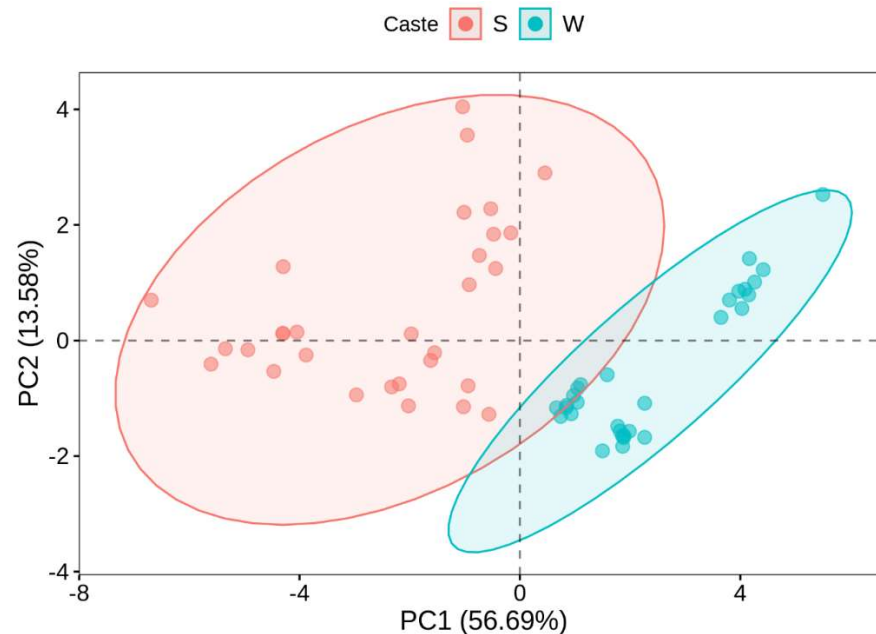


Figure 4.2. Principle component analysis of CHCs of soldiers (red) and workers (blue) in *C. formosanus*. A total of 15 CHCs were used in the analysis and each compound was normalized to total CHCs (n = 30 per caste, with 10 samples each of the three colonies tested).



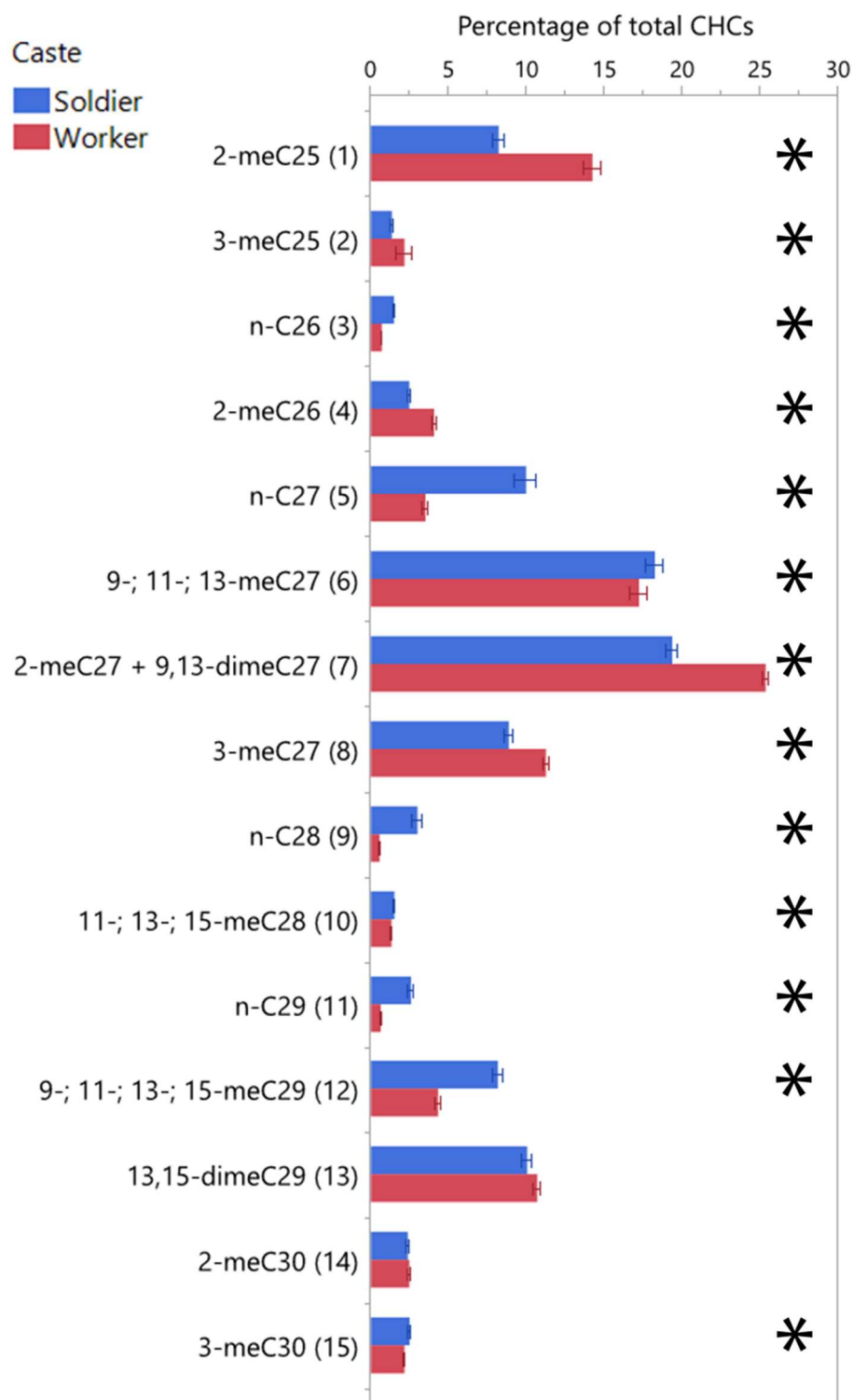


Figure 4.3. Mean relative percentage ( $\pm$  SEM) of 15 CHC peaks from workers and soldiers. Asterisks indicate significant difference between castes ( $P < 0.05$ , GLMM,  $n = 30$ ). Peak numbers correspond with peaks listed in Figure 4.1 and Table 4.1.

#### 4.4. Discussion

We analyzed 15 peaks in the CHC profiles of *C. formosanus* workers and soldiers collected from Louisiana, 13 of which occurred in significantly different relative proportions between the two castes. Haverty et al (1996) compared CHC profiles between castes of *C. formosanus* and listed 39 peaks, 12 of which are reported here including peaks 1 through 12 (Table 4.1, Figures 4.1 and 4.3). Of these 12 peaks, significant differences between worker and soldier percentage matched for only three peaks, P4, P5, and P8 (2-meC26, *n*-C27, and 3-meC27). The differences between the previous and this study may be partially attributed to different geographic populations and seasonal variations. Future work looking at how caste-specific CHCs fluctuate based on environmental conditions, such as season, location, diet, humidity, etc., will allow us to identify relationships in relative percentage of CHCs within workers and soldiers that may provide insight on how they discern caste. Special focus may be set on 2-meC26, *n*-C27, and 3-meC27, as these compounds showed consistent differences between the two castes in different experiments.

Workers must be able to distinguish each caste within their colony to ensure proper care and husbandry, but the method through which *C. formosanus* workers recognize caste is not entirely known. The relationship between caste and CHC profile is not as clear in *C. formosanus* as it is in some other termite species like *R. flavipes* (Darrouzet & Labedan 2014; Funaro et al., 2018; Sun et al. 2020) or some hymenopterans (Wagner et al. 2000; Mill et al. 2009). *C. formosanus* CHC profiles are variable depending on environmental conditions (Prestwich 1985; Haverty et al. 1990; Haverty et al. 1996; Florane et al. 2004; Perdereau et al. 2010). Colony age can also affect CHC profiles. In

*C. gestroi*, caste can be distinguished by CHC profiles in a young colony, but as colonies grow, caste CHC profiles homogenize (Gordon et al. 2020).

The role of CHCs has received much attention in termites (Howard et al. 1978; Blomquist and Jackson 1979; Howard et al. 1982; Hadley 1984; Singer 1998, Su & Haverty 1990, Chouvenc & Su 2017), and while *C. formosanus* worker and soldier CHC profiles have been compared (Haverty et al. 1996), the behavioral significance has not been explored. Our work showed the differences between workers and soldiers in their relative abundances of 2-meC26, *n*-C27, and 3-meC27 were like those reported by Haverty et al. (1996), so these compounds should receive special focus in future studies. It is also important to investigate other chemical cues, such as volatiles, that may complement or synergize with CHCs for termites to recognize caste, along with behavioral assays to determine the biological activities of the compounds. These studies will help us to fully understand the complicated chemical interactions that take place in eusocial environments.

## Chapter 5. Conclusions and discussion

Termites are destructive urban pests that have become established globally and contribute to billions of dollars in destruction every year. *C. formosanus* is one of the most destructive termites contributing to \$1 billion in structural damage in the United States (Rust & Su 2012), an annual figure that has likely increased in the past 20 years. It is important to understand the behavioral and chemical ecology of this species for better management. These studies can also provide information to understand the evolution of eusociality in Blattodea, an important topic that does not receive as much attention as that in Hymenoptera.

In Chapter 2, we did not observe any differences in exploratory foraging behavior or food consumption between foraging groups with the presence of different numbers of soldiers (0, 2, 10, or 30) (Figures 2.2–2.4). The findings are different from previous studies, in which soldiers influence on foraging has been observed in several termite species, including *Nasutitermes costialis* (Trianello 1981), *Reticulitermes* spp. (Janowiecki & Vargo 2022), and *Coptotermes intermedius* (Olugbemi 2011; 2012). Further studies are required to determine what, if any, influence *C. formosanus* soldiers have on workers in different social contexts.

The presence or absence of royals has been shown to change the aggression level of workers and soldiers of *R. speratus* (Konishi & Matsura 2020), and while this has not been demonstrated in *C. formosanus*, these types of social context may influence the behavioral responses of workers and soldiers in a foraging arena. Investigation of this behavior with other species of termites, especially closely related species *C. gestroi*, may

further provide insight into the multifunctional roles of soldiers and social interactions in *Coptotermes*.

In Chapter 3, we observed significant differences in termite choice assays when workers were exposed to major components of the soldier defensive secretion. Workers at some time points avoided filter paper treated with lignoceric or hexacosanoic acid in favor of the control paper which was treated with solvent only (Figure 3.1 A–B). When lignoceric and hexacosanoic acid solutions were mixed, workers avoided the mix treatment at every time point (Figure 3.1 C). There was some avoidance behavior observed with the filter paper treated with the crude extract, and this effect reduced as time went on (Figure 3.1 D).

Volatile fatty acids and fatty acid esters are known repellants to some hymenopterans and dipterans (Howard et al., 1982; Henderson et al., 1991; Henderson et al., 1993). While there are no reported instances of enemy repellant or toxicity for *C. formosanus* (Mill, 1983; Waller and La Fage, 1987; Mao et al., 2005), the major components of the defensive secretion are free fatty acids (Chen et al., 1999) and may have other activities against some other enemies. Additionally, *C. formosanus* defensive secretion contains a lysosome (Hardt et al., 2003) and Kazal-type serine protease inhibitor (Negulescu et al., 2015). Though their functions have not been investigated, they may provide protection against bacterial or fungal infection (Negulescu et al., 2015). It is likely that the defensive secretions of *C. formosanus* have other unidentified components, and its ubiquity within the colony suggests it may be responsible for other unknown physiological and behavioral effects in both *C. formosanus* and the organisms that interact with them.

In Chapter 4, we report quantitative differences between several CHC peaks between workers and soldiers (Table 4.1, Figures 4.1 and 4.3). Peaks 1, 4, 7, and 8 were all relatively more abundant in workers than soldiers, and peaks 3, 6, 9, 10, 11, 12, and 15 were more relatively abundant in soldiers. Haverty et al (1996) reported some of the same hydrocarbons (peaks 1–12), with differentially regulated CHCs between worker and soldier castes partially overlapping with findings in this study.

Haverty et al (1996) also reported more than twice as many peaks (32) than we did in this study (15). This difference may result from sampling technique or the fact that the termites were collected at different times in different states (~27 years apart) (New Orleans, Louisiana for this study and Ouahu, Hawaii for Haverty et al. (1996)). Further studies may focus on differences in CHC profiles across different populations of *C. formosanus* from native and introduced ranges, at different seasons, and at different colony ages to determine a pattern of relative CHC differences between workers and soldiers. It is also important to investigate other chemical cues, such as volatiles, that may complement or synergize with CHCs for termites to recognize caste, along with behavioral assays to determine the biological activities of the compounds.

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## **Vita**

Joseph Edward McCarthy, born in Syracuse, New York, worked as a waiter while completing his bachelor's degree in biology from the State University of New York at Oswego. Originally, his interest in entomology took him on several zookeeping internships. Influenced by the guidance of his phenomenal undergraduate professors and the books of Dr. Edward Wilson, he decided to apply to Louisiana State University to study one of the most fascinating behavioral adaptations in animals: eusociality. Joseph discovered while working on his graduate degree that his favorite aspect of academia was scientific communication, specifically to non-scientists, and upon completion of his master's degree he will pursue a career in extension and outreach. He plans to receive his Masters in December, 2022.