TRANSFER EFFICIENCY BY THE SUBTERRANEAN TERMITE *RETICULITERMES FLAVIPES* (ISOPTERA: RHINOTERMITIDAE) OF FOOD-BORNE BAIT FORMULATIONS CONTAINING BENZOYLPHENYL UREA CHITIN SYNTHESIS INHIBITOR

by

JENNIFER LYNN LEWIS

(Under the Direction of Brian T. Forschler)

ABSTRACT

Experiments were conducted exposing the eastern subterranean termite [*Reticulitermes flavipes* (Kollar)] to one of five chitin synthesis inhibitors (CSIs); these included diflubenzuron, hexaflumuron, lufenuron, noviflumuron, and one experimental CSI in commercially available bait matrixes. Following CSI exposure, termite donors (D) were combined with naïve nestmates (R) and horizontal toxicant transfer studied in laboratory bioassay. Various D:R ratios included 20:0, 15:5, 10:10, 5:15, and 1:19 and mortality accessed daily for 68 days. All CSIs tested were efficiently transferred over the time period examined as evident by termite mortality of non-exposed nestmates, except for diflubenzuron at the lowest ratio. Behavioral responses must also be considered when evaluating pesticide efficacy. Observations of CSI impacts in *R. flavipes* were recorded and cannibalistic acts quantified. I reported the frequency of dead and moribund showing evidence of CSI intoxication, including the previously described "jackknife" pose and an additional physical deformity, I termites "body curl". Cannibalism of CSI exposed termites was not as frequent as termites from nontreated controls but due to efficient toxicant transfer,

equivalent mortality levels were recorded. Termiticides can target either the insect or their microbial symbionts. I quantified the anaerobic protists from termites exposed to CSIs and provided evidence they were negatively impacted after only 3 days.

INDEX WORDS: Eastern Subterranean Termite; Termite Control; Symbiotic Protist Communities; Proctodeal Trophallaxis; Cannibalism, Horizontal Transmission.

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B.A., University of Louisville, 2001

M.S., University of Georgia, 2003

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2008

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December 2008

DEDICATION

I would like to dedicate this dissertation to my grandmother, Martha Blackburn. It seems like ages ago I gave you my word I would pursue a Ph.D. degree and I never forgot that promise.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my major advisor, Dr. Forschler. He never gave up on me even when I had already done so myself. Without his encouragement and guidance, I would never have grown into the person I am happy to say I have become.

I would like to express my sincere gratitude and heartfelt thanks to my committee members, Drs. Champagne and Noblet, who provided me with constructive advice and support.

I would also like to acknowledge the help of all the support staff and secretaries. Whenever I walked into the office frustrated with a problem, I always left with a smile and a sigh of relief. Thank you.

I am thankful for Dr. Charles V. Covell jr., who was instrumental in my initial pursuit of entomology. His time, engaging classes, lab space, and encouragement in the academic field of entomology will forever be greatly appreciated.

Last but not least, my parents who taught me in one way or another, perseverance.

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CHAPTER 1

INTRODUCTION

The structure of this dissertation is written in manuscript style. This research illuminates aspects of the mode of transfer, impact, and a comparative assessment of several chitin synthesis inhibitor (CSI) active ingredients (AI) on *Reticulitermes flavipes* (Kollar). The first chapter (Chapter 3) investigates the transferability of five food borne toxicants. A comparison of commercialized CSIs baits formulations and subsequent mortality levels determined. This work will be submitted to *The Journal of Economic Entomology*. The second chapter (Chapter 4) considers the impacts of cannibalism in toxicant transfer on *R. flavipes*. This chapter of my dissertation provides empirical studies on toxicant transfer by cannibalism. The third chapter (Chapter 5) will be submitted to *Environmental Entomology* and examines CSI impacts other than molting inhibition based on symbiotic protist communities present in the termite hindgut. These results provide evidence the protist community was negatively impacted by CSI exposure and that some protist species might be more influenced than others might. Finally, the last chapter briefly summarizes my results.

CHAPTER 2

A REVIEW OF THE SCIENTIFIC LITERATURE ON TRANSFER OF FOOD-BORNE CHITIN SYNTHESIS INHIBITING TOXICANTS AND TERMITE BAITING EFFICACY

2.1 Introduction

Termites are from the order Isoptera (iso = same, ptera = wings). There are over 2,600 described termite species from seven recognized families worldwide (Kambhampati and Eggleton 2000). Of these, about 50 termite species are found in the United States with eighteen indicated as structural pests (Thorne and Forschler 1999). Isoptera are divided into the lower and higher termites based on symbiotic microbes. Lower termites have both flagellated protists and bacteria while higher termites rely solely of bacteria to aide in cellulose digestion.

Termites can be found inhabiting soil or wood, which also serve as a food resource. Information on basic termite biology is incomplete in part due to the great difficulty of sampling (Sands 1972, Haverty and Nutting 1975). Termites are cryptic in nature and have a flexible life history making them a challenge to sample (Nutting and Jones 1990).

Termites have an important impact on ecosystems by influencing physical, chemical, and structural attributes of soils (Lee and Wood 1971, Wood and Sands 1978). Their populations can exceed 6000/m^2 in the tropics, but termite numbers and diversity decline with distance from the equator (Eggleton et al. 1996, Eggleton 2000). However, what termites are most noted for is their capacity to cause considerable structural damage to buildings and homes.

2.2 Termite Biology

Termites are eusocial insects; they can have overlapping generations, cooperation in brood care, with some individuals sterile and others specialized for reproduction (Wilson 1971). Termites display distinct castes including the reproductive, soldier, and worker forms, with each caste performing a role within the colony (Noirot and Noirot-Timothee 1969). Reproductives maintain colony populations through egg production and the soldier caste guards and protect the colony from invaders. Workers, the most numerous caste in a colony, perform many tasks such as building and maintaining extensive galleries, caring for brood, foraging for and consuming food, and feeding other colony members (McMahan 1969, Honigberg 1970).

Dead wood, live plants, and leaf litter are the major food resources of lower termites: Hodotermitidae, Termopsidae, Mastotermitidae, Kalotermitidae, Serritermitidae, and Rhinotermitidae (Lee and Wood 1971, Waller and LaFage 1987). Termites ingest wood fragments, macerating and mixing them with salivary gland secretions that include endogenous cellulases (Watanabe et al. 1997, Nakashima et al. 2002) that hydrolyze cellulose polymers to glucose (Inoue et al. 1997). Soluble nutrients such as glucose are absorbed from the midgut; however, it has been estimated that about 70 percent of cellulose digestion and absorption takes place in the dilated portion of the termite hindgut, or paunch (Inoue et al. 1997). The paunch, filled with bacteria and protists, accounts for up to 61 percent of a termite's hindgut weight (Odelson and Breznak 1983). These obligate symbionts aid in cellulose digestion, creating a unique system important for termite health (Cleveland 1924, 1925a-c, 1928, Trager 1934, Hungate 1938, 1939).

Wood is a nutritionally poor food source and contains a high C:N ratio (~400:1) (Cowling and Merrill 1966). The nitrogen content of wood is from 0.03-0.1% nitrogen and is

too low for termite nutritional needs (Breznak and Brune 1994). To compensate, termites have symbiotic bacteria that can either fix atmospheric nitrogen or recycle nitrogenous waste (Hungate 1941, Potrikus and Breznak 1981). Termites can cannibalize nestmates to obtain their required nitrogen for protein synthesis (La Fage and Nutting 1978).

Food availability has a significant impact on termite growth (Lenz 1994, Korb and Lenz 2004). Termites can also distinguish among variable resources and selectively forage for higher quality food (Waller and La Fage 1987, Lenz 1994). Foragers search and colonize a diffuse network of several food resources (Moore 1969, Wilson 1971) creating decentralized nest system and respond quickly to a 'new' food source, produce large number of young, and disperse.

2.3 History of Termite Control

In the early 19th century, termite control consisted of managing building practices (Snyder 1927, Kofoid 1934, Brown et al. 1946). This included trying to minimize environmental conditions favored by termites like moisture and wood debris next to buildings. It was recommended that building materials should not be a preferred food substrates by termites nor in direct contact with the soil. However, in the last 50 years, termite control tactics have instead relied more heavily on chemical methods to protect urban dwellings, consisting of chemical fumigants, soil termiticides and or wood preservation.

The first manuscript to obtain some notoriety of using the termite's own behavior to transfer bait toxicant was by Randall and Doody (1934). As noted in this manuscript, the initial report of applying a slow-acting bait toxicant to termite galleries was in 1916 by Van Zwaluwenberg and noted the idea was taken from another entomologist (Randall and Doody

1934). However, it was not until the 1930s poisonous dusts were recognized as having possible health risks.

Insecticidal ground treatments were used during the 1930s-1950s with the goal to block all potential routes of termite entry into a structure by either killing or repelling them (Randall and Doody 1934). Soil treated with liquid termiticides forms a chemical barrier between the structure and termites in the soil. Treatments included sodium arsenite, trichlorobenzene, DDT, pentachlorophenol, creosote, and ethylene dibromide (Su and Scheffrahn 1998). Then near the early 1950's, termiticide barrier treatment expanded to include other organochlorines, like chlordane and heptachlor. These insecticides bind gamma amino butyric acid (GABA)-gated chloride channel thus blocking the influx of chloride ions of both sensory and motor neurons (Bloomquist 1994). The treated insect remains in a state of uncontrolled excitation leading to lethal spasms and seizures. Registrations of organochlorines were removed from use in the U.S. in 1987 based on the potential carcinogenicity and persistence in the environment (Peakall and Lincer 1970).

Termite population management typically exceeds over 5 billion dollars worldwide (NPMA 2006). Previous control methods did not incorporate termite behavior or their natural history, and were largely ineffective (Su and Scheffrahn 1988). Commercial termite management methods still rely on termiticide treated soil but these registered compounds (permethrin, fipronil, imidacloprid) are costly and do not provide persistent protection. Instead, termite management strategies incorporate baiting systems by targeting the pest. Bait delivery is introduced through using the termite's own behavior, such as grooming and the transfer of food (Suárez and Thorne 2000). This horizontal transmission of insecticides is utilized in termite population management on the premise foragers pick up a toxic dose that is slow acting (w/in

weeks) and nonrepellent, thus, providing the potential of wide distribution within a termite colony before the onset of acute toxicity or avoidance behavior (Myles 1996).

2.4 Chitin Synthesis Inhibitors

Chitin is one of the most abundant organic compounds found in nature, second to cellulose and similar in structure. They are both highly stable molecules resistant to hydrolysis by many solvents and serve as structural components supporting cell and body surfaces. Chitin is a homopolymer of covalent β-1, 4 linkages of N-acetyl-D-glucos-2-amine units and composes the cell wall of fungi and exoskeleton of arthropods. The synthesis of chitin involves a complex series of biochemical pathways and is associated with proteins and proteoglycans that provide this structure some flexibility.

The exoskeleton of insects provides protection from desiccation, muscle attachment and covers the body. The major structural component is chitin (25-50%) and is synthesized by ectodermal cells in the trachea, salivary glands, epidermis, foregut, and hindgut (Andersen 1979, Cohen 1987). The midgut extracellular membrane also contains chitin (3-13%; Peters 1992) forming a peritrophic matrix that helps protect the insect from ingested abrasive particles and microorganisms (Lehane 1997, Wang and Granados 2001). In addition, insect eggs are covered by a chitinous shell, the chorion, important in egg viability (Cohen 1993).

Chitin synthesis inhibitors (CSIs) are insect growth regulators that interfere with an insect's physical development and cuticle sclerotization during growth and reproduction. These benzoylphenyl ureas have a high degree of selectivity and low mammalian toxicity and were discovered by scientists at Philips-Duphar, Netherlands in the 1970s (Van Daalen et al. 1972, Mulder and Gijswijt 1973, Wellinga et al. 1973a, 1973b). Initially they were trying to develop a

weed control agent but found the product was more effective as an insecticide showing a delay in toxicity when the insect next molted (Verloop and Ferrell 1977, Retnakaran and Wright 1987). Because CSIs interfere with the polymerization of chitin, this mode of action has been targeted for control o of several different insect pests.

CSIs cause abnormal deposits of endocuticle that accumulate during molting (Mulder and Gijswijt 1973), specifically uridine diphospho-N-acetylglucosamine monomers thus stopping chitin synthesis (Verloop and Ferrell 1977, Deul et al. 1978, Eck 1979, Hajjar and Casida 1979). This creates a weakened cuticle and causes mortality when the procuticle is subjected to the stresses of ecdysis and cuticular expansion (Dean et al. 1998). Observations of CSI toxicity also include mortality in the absence of metamorphosis and include swollen appendages, decrease in locomotion, inability to eat due to dislocation of mandibles, malformed or absent peritrophic matrix, as well as suppressed fecundity and egg viability (Grosscurt and Jongsma 1987, Retnakaran and Wright 1987, Marco et al. 1998, Morales-Ramos et al. 2006).

The first commercially available CSI bait system marketed was the Sentricon® Termite Elimination System by Dow AgroSciences (DowElanco, Indianapolis, IN) and the University of Florida in 1990. The active ingredient in Sentricon® bait is hexaflumuron [N-[[[3, 5-dichloro-4-(1,1,2,2-tetrafluoroethoxy) phenyl]amino] carbonyl]-2,6-difluorobenzamide] and is characterized as a slow acting, nonrepellent toxicant that can be effectively transferable from treated to non-treated termites (Sheets et al. 2000, Haagsma and Rust 2005). Studies have shown various degrees of termite management success with hexaflumuron (Forschler and Ryder 1996, Getty et al. 2000, reviewed in Su 2003, Vargo 2003, Karr et al. 2004). Efforts to improve CSI efficacy by examining other benzoylphenyl urea insect growth regulators for termite population management in field studies have been conducted (Smith et al. 2001, Sajap et al. 2005, Cabrera and Thoms

2006). However, it is important to consider termite behavior when evaluating the efficacy of a termiticide (Su et al. 1982, Haverty et al. 1989, Forschler and Jenkins 2000).

2.5 Toxicant Transfer

The transfer of nutrients, pheromones, and symbionts among termite nestmates is an essential behavior for their health and growth (McMahan 1969, Peppuy et al. 1998). Workers acquire and then transfer food to dependent nestmates by food sharing (Grassé and Noirot 1945, McMahan 1969, Noirot and Noirot-Timothee 1969) in the form of allofeeding stomodeal (contents from mouth) and/or allofeeding proctodeal food (hindgut contents) (Whitman and Forschler 2007).

An important aspect of termite biology includes the acquisition of symbiotic microbes within the termite hindgut (Cleveland 1924, 1925a-c, 1928, Trager 1934, Hungate 1938, 1939). Termites are not born with them, but instead must be faunated by nestmates. Following molting, these microorganisms are shed along with the exuvia (Grassé and Noirot 1945) requiring the recently molted individual to feed on exudates from the anus of another termite (Grassé and Noirot 1945) to regain the hindgut microorganisms lost.

Sharing of nutrients by proctodeal donation is the dominate route of toxicant transfer (Whitman and Forschler 2007). For efficient horizontal transmission from an exposed termite to a naïve nestmate, a termite worker must consume a meal from a toxicant-bait, and then move some distance from the feeding site. This toxicant must remain unaltered within the alimentary canal and then elicited for a food donation.

2.6 Summary and Future Studies

The interference of an insect's physical development as caused from CSIs is one tool used in termite pest management. Current practices include bait toxicant delivery that is introduced through ingestion by termite workers, and then dependent on the transfer of active ingredient by "donors" to naïve nestmates (Randall and Doody 1934, Beard 1974, Rosengaus et al. 1986, Suárez and Thorne 2000, Nalepa et al. 2001). CSIs can cause death by the inability to separate from exuvia following molting (Su and Scheffrahn 1993, 1996, Karr et al. 2004). However, the method of CSI food-borne toxicants transfer, be it behavioral, cannibalism, coprophagy, or grooming is still not known.

Other detrimental impacts caused by CSI toxicity must also be investigated (Retnakaran and Wright 1987, Marco et al. 1998, Perrott 2003, Morales-Ramos et al. 2006). There are several thousand symbiotic protists in the hindgut of termites' and some protist species are more sensitivity to oxygen (Cleveland 1925b, 1925c, Grosovsky and Margulis 1982). It is possible a decrease in chitin in the lining of the hindgut could increase oxygen exposure, thus killing strict anaerobes.

Investigating insecticide uptake and termite-to-termite transfer would help in understanding the movement of this chemical throughout termite populations but ultimately it must be tested in field studies. Investigations of nonrepellent termiticides in both laboratory and field investigations are essential in developing effective termite population management.

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CHAPTER 3

TRANSFER EFFICIENCY BY THE SUBTERRANEAN TERMITE *RETICULITERMES FLAVIPES* (ISOPTERA: RHINOTERMITIDAE) OF FIVE COMMERCIAL TERMITE BAIT FORMULATIONS CONTAINING BENZOYLPHENYL UREA CHITIN SYNTHESIS INHIBITOR[1]

[1] Lewis, J.L. and B.T. Forschler. To be submitted to *The Journal of Economic Entomology*.

3.1 Abstract

Eastern subterranean termite, *Reticulitermes flavipes* (Kollar) workers were exposed for 7 d to one of five chitin synthesis inhibitors (CSIs) including diflubenzuron, hexaflumuron, lufenuron, noviflumuron, and one experimental CSI in commercially available bait matrixes. Following a 7 day exposure period, termite donors (D) were combined with naïve (not exposed) termite recipients (R) at five D:R ratios (20:0, 15:5, 10:10, 5:15, and 1:19) and mortality determined daily up to 68 d. Lethal time and percent mortality data show efficient transfer at all D:R ratios for all CSI's tested, except diflubenzuron at 1:19. Despite the data indicating efficient transfer of lufenuron in bioassay, this material may not be effective in field use based on behavioral observations that include limited movement by donors. We also report frequency of visible evidence of CSI intoxication, including the previously described "jackknife" pose and an additional physical deformity, we termed "body curl". The implications these data and observations have for laboratory evaluation and field population management using commercialized termite baiting systems are discussed.

3.2 Introduction

Chitin, an amino-polysaccharide (poly-β-(1, 4)-N-acetyl-D-glucosamine), is a major structural component of insect cuticle synthesized in ectodermal cells of the trachea, salivary glands, epidermis, foregut, and hindgut (Andersen 1979, Cohen 1987). Benzoylphenyl urea insecticides are considered chitin synthesis inhibitors (CSI) that cause death by interfering with cuticle sclerotization during molting (Cohen 1987). CSI's display arthropod specificity and delayed toxicity and have been examined as an alternative to neurotoxin insecticides (Verloop and Ferrell 1977, Retnakaran and Wright 1987). Slow acting toxicants have limited utility in

crop protection but provide advantages when used in a pesticidial baiting program (Williams and Lofgren 1981, Su et al. 1982, Reierson 1995).

Termite baits, containing CSI active ingredients that are readily consumed within a range of concentrations, and display delayed toxicity were commercialized for termite control in the late 1990's using (Su et al. 1982, Su et al. 1987, French 1994, Su 2003, van den Meiracker et al. 2002). Termiticidial agents introduced into a termite population can be distributed through eusocial behaviors such as grooming, food sharing, and cannibalism (Randall and Doody 1934, Beard 1974, Su and Scheffrahn 1996, Peppuy et al. 1998, Ibrahim et al. 2003, Hu 2005, Haagsma and Rust 2005). The CSI active ingredients used in U.S. EPA registered termite bait systems include diflubenzuron 0.25% active ingredient (AI) (Whitmire Micro-Gen, St. Louis, MO), hexaflumuron 0.50% (Dow AgroSciences, Indianapolis, IN), noviflumuron 0.50% (Dow AgroSciences, Indianapolis, IN), and lufenuron (0.15%; Syngenta Corporation, Greensboro, NC). These CSIs are analogs differing in halogen substitution or side-chain modification on the phenyl ring and it is assumed they have the same mode of action, impact on termite behavior, transfer efficiency, and dose response (Fig. 3.1).

We used laboratory bioassay to record mortality associated with each of five CSI-containing termiticidal baits using the eastern subterranean termite, *Reticulitermes flavipes* (Kollar). Efficiency of CSI transfer was measured using five donor-to-recipient (D:R) ratios, along with notation of cannibalism and physical deformities. The null hypothesis was that all CSI active ingredients would provide the same profile of mortality and be equally efficacious in transfer between nestmates.

3.3 Material and Methods

Insects and Chemicals. Five populations of *Reticulitermes flavipes* (Kollar) were collected from field sites, separated by at least 100 m, in Whitehall Forest, Clarke County, Georgia. Termites were identified to species using published keys to the soldier caste (Scheffrahn and Su 1994). Termites were collected using moistened corrugated cardboard and placed into a plastic container (26.99 X 19.37 X 9.52 cm) with weathered pine wood slats (approx. 12.5 X 2.54 X 0.2 cm) in complete darkness inside an environmental chamber (27° C, ≥90% RH) until used in bioassays (Forschler and Townsend 1996). Termites used as donors were obtained by placing 300 workers (4^{th} instar or older) in a plastic Petri dish (100X25 mm diameter) with 20 grams of sand moistened with 3.2-ml of distilled water and a known weight (6.5 ± 1.5g) of commercially available α-cellulose tablet containing one of the following treatments; no CSI (control), diflubenzuron 0.25% (Whitmire Micro-Gen, St. Louis, MO), hexaflumuron 0.50% (Dow AgroSciences, Indianapolis, IN), noviflumuron 0.50% (Dow AgroSciences, Indianapolis, IN), and a noncommercial experimental CSI 0.50% AI (Whitmire Micro-Gen, St. Louis, MO), for 7 d. The lufenuron (Syngenta Corporation, Greensboro, NC) treatments were presented on 2 ± 0.2 g corrugated cardboard at 0.15% AI as this is the bait matrix of the commercial product. Termite feeding is highly variable (Forschler 1996) and bait consumption were not determined.

Toxicant Transfer. Donor termites were placed in a plastic Petri dish (65X15 mm diameter) containing filter paper (Whatman #1, 55 mm diameter) moistened with distilled water. Nestmate workers from the same population (recipient termites) were added to provide various donor:recipient (D:R) ratios for a total of 20 workers per Petri dish. Recipient termites (R) were treated as described for the donor termites (D) except R were exposed to non-treated α-cellulose

tablets and marked with DecoColor Paint Marker (Uchida American DecoColor Paint marker) to differentiate them from D. Several D:R ratio's were tested; 20:0, 15:5, 10:10, 5:15, and 1:19. Each D:R was replicated at least 5 times from each termite population.

Data Collection. All dead and moribund termites were removed daily and the condition (whole or missing body parts) of each cadaver was noted. Individuals with over half of an appendage missing were classified as cannibalized. We set a limit of <20% mortality in the corresponding control as the benchmark for including a replicate in the data analysis. Information on the presence of termites found in the "jackknife" position (Su and Scheffrahn 1993) characterized by the head and last abdominal segments being in close proximity because the thorax and first abdominal segments were raised (Fig. 3.2A). Termites in the jackknife pose also displayed visibly wrinkled cuticle near the tip of the abdomen (Fig. 3.2A). We also observed termites that arched in the opposite direction that we termed the 'body curl' position (Fig. 3.2B). The body curl was not characterized by any deformity of the cuticle. The body curl position was observed only in moribund termites because in death these termites 'relaxed' into a normal straight position.

Statistical Analysis. Mortality data were adjusted using Abbott's formula (1925) and compared using SAS-JMP (version 7.0) statistical software (2007 SAS Institute, Inc., Cary, NC) by treatment, ratio, and time, while controlling for the effects of termite population. Termite mortality was evaluated with GLM analysis of variance (ANOVA) and Tukey-Kramer honestly significant difference (HSD) test for multiple mean comparisons ($\alpha = 0.05$). Mortality data were also subjected to probit regression to obtain lethal time (LT) estimates. If the confidence interval (CI) ($\alpha = 0.05$) of the LT values did not overlap, they were considered significantly different. The number of body parts cannibalized were evaluated at the end of census period using analysis

of variance (ANOVA). Tukey-Kramer honestly significant difference (HSD) test ($\alpha = 0.05$) was performed to identify differences.

3.4 Results

Meaningful comparisons within and between treatments were complicated because about half of the mortality curves provided slopes that were not equivalent within and between treatments for all of the D:R ratios tested (Table 3.1). If we ignore the model assumption of similar slopes (Finney 1971) and compare mortality curves based on CI overlap, as is common in the literature (Retnakaran and Wright 1987, El Saidy et al. 1989, Su and Scheffrahn 1991, Sheets et al. 2000, Medina et al. 2003), the LT_{90} values were similar from all CSI's tested for the first two D:R's (20:0, 15:5) (Table 3.1). The 10:10 D:R indicated no difference between the LT_{90} estimates for the experimental CSI, hexaflumuron, noviflumuron and lufenuron, that lufenuron and diflubenzuron were not different but, diflubenzuron where longer than the other three (Table 3.1). The establishment of statistical significance, determined by CI overlap, in the aforementioned estimates varied by one to two days which may be argued to be biologically insignificant. However, diflubenzuron clearly separates statistically (and, likely, biologically) from all the other CSI's at the 5:15 and 1:19 D:R's with a longer LT_{90} estimate (Table 3.1). Lufenuron, at the 1:19 D:R, also statistically separated from the remaining chemistries (Table 3.1).

The LT_{50} data indicate a similar trend when comparing CSI treatments (Table 3.1). The LT_{50} data examined within treatments implies that noviflumuron is transferred most efficiently as signified by the lack of statistical difference. The difference in LT_{50} values for the hexaflumuron and experimental CSI D:R comparisons were statistically the same despite ranging between 4-5

days (Table 3.1). The lufenuron LT_{50} data display a clear statistical trend toward less efficient transfer as the number of donor's decrease as well as diflubenzuron, especially at the two lower D:R ratio's (Table 3.1).

The percent mortality data, by treatment, from the 20:0 ratio provided the benchmark for efficacy assuming all exposed termites consumed the respective toxicant during the 7 days they were confined with the treated matrix (Fig. 3.3A). All the CSI treatments, at 27^{o} C, provided sufficient mortality (>97%) by Day 68 to be considered efficacious if the targets fed on the bait (Fig. 3.3A; F=589.60; df=11; p<0.0001). The mortality data in the remaining D:R's (F=272.62, F=221.88, F=76.79, F=73.82, respectively) demonstrate the effective transfer of CSI's in this confined bioassay system (Figs. 3.3B-E; df=11; p<0.0001). Hexaflumuron, noviflumuron, and experimental CSI provided equivalent mortality at Days 42 (df=20; F=35.65) and 68 (df=15; F=29.48) (Fig. 3.3A-E; p<0.0001). Lufenuron provided higher mortality on Days 7 (F=5.41) and 14 (F=6.02) in the 20:0 D:R treatment indicating a quicker affect compared to the other CSI's that was not realized in the lower D:R's (Fig. 3.3; df=11; p<0.0001). Lufenuron mortality was also statistically similar to the three previously mentioned CSI's at Days 21 and 42 for all D:R's except 1:19 (df=11; F=73.82; p<0.0001) indicating a reduction in transfer efficiency at low donor ratio's (Fig. 3.3E). The diflubenzuron treatment produced statistically lower mortality by Day 68 in the 10:10 and 5:10 D:R's (df=7; F=20.21; p<0.0001) and at Day 42 in the three lowest D:R's, 10:10, 5:10, 1:19 (df=9;F=12.67; p<0.0001), indicating reduced transfer efficiency compared to the other CSI's (Fig. 3.3).

Observations from Cadaver Removal. D:R ratio was not an important variable in the observed condition/treatment of the dead (df=47, 308; F=1.08; p=0.3603) and therefore data were pooled by CSI treatments. CSI exposure had a significant effect on observed acts of

cannibalism (Fig. 3.4), with most (65-75%; N=60) cadavers, found during the daily procedure of removing the dead termites, intact (not missing body parts). The number of dead removed from the controls by contrast provided 24 ± 32% (N=60) intact cadavers (Fig. 3.4; df=5, 47; F=52.67; p<0.0001). Body parts most likely to be missing in the control included the abdomen (13 ± 18%; F=13.58), head (18 ± 15%; F=18.03), or thorax (9 ± 10%; F=14.53) and were at least 2 times as likely as in the CSI treatments (Fig. 3.4; df=5, 47; p<0.0001). Controls also were more likely to have legs (11 ± 11%; F=5.14; p=0.0002) and antennae (17 ± 15%; F=6.19; p<0.0001) missing, compared to all CSIs treatments that averaged 6 ± 5% and 9 ± 7%, respectively (Fig. 3.4; df=5, 47). Burial of cadavers was (3 ± 6%; N=360) uncommon and did not differ between treatments (Fig. 3.4; df=5, 47; F=1.30; p=0.1004). Instances of individuals being unaccounted for and presumed completely cannibalized were rare averaging 3% (N=360) or less over 68 d (Fig. 3.4; df=5, 47; F=2.78; p=0.0179) for non-treated controls and treatments.

We observed two physical deformities displayed by moribund termites exposed to CSIs, termed “jackknife” (Su and Scheffrahn 1993) and “body curl” (Fig. 3.2). Within treatments the occurrence of either jackknife (F=1.0832; p=0.3603) or body curl (F=1.3732; p=0.2559) was not different between D:R ratios (Fig. 3.5). The jackknife pose was seen in termites exposed to all CSI's, with the exception of the experimental CSI, and was most common in the diflubenzuron treatment (Fig. 3.5; F=17.7874; p<0.0001). Diflubenzuron provided comparable frequencies of both jackknife and body curl 38 ± 15% and 39 ± 18%, respectively (F=0.0181; p=0.8936). All other CSI's provided a higher frequency of body curl (>60%) with hexaflumuron providing the highest incidence (Fig. 3.5; F=60.1281; p<0.0001).

3.5 Discussion

Maintaining healthy termites in bioassay is essential when comparing experimental results (Lenz and Williams 1980), and we established a threshold of less than 20% mortality in the control group before including a replicate in our data analysis. Maintaining termites in small plastic containers for months is difficult as indicated by publications such as Vahabzadeh et al. (2007) who reported 70% control mortality at Day 45. Of the several subterranean termite/CSI studies published, only three had less than 20% mortality in the controls (van den Meiracker et al. 2005, Karr et al. 2004, King et al. 2005).

This was the first study to compare the transfer of several commercialized CSI baits on different termite populations. Of the studies with "healthy" controls, termite mortality from our CSI treatments corresponded well with van den Meiracker et al. (2005) (25 and 30° C; continuous exposure), King et al. (2005) (21° C; 14 day exposure) and Karr et al. (2004) (26° C; 7 day exposure) that reported about 80% mortality by day 42 (Fig. 3.3A).

Temperature is one of the factors that can influence frequency of molting and therefore time to mortality attributable to CSIs. Subterranean termite workers can take 50-125 days between molts with temperatures ranging from 30-18° C (Weesner 1956, Buchli 1958, van den Meiracker et al. 2002, Swoboda and Miller 2005, Raina et al. 2008). When investigating the impacts of a slow acting compound, it is essential to conduct a study at a temperature that will permit observation of mortality in a timely manner (van den Meiracker et al. 2002). Despite the fact that the annual soil temperature of the Georgia Piedmont averages 22° C at a depth of 20 cm (NRCS 2007, GeorgiaWeather.net 2008) we chose, for comparative analysis, a higher temperature (27^{o} C) in order to observe mortality in a time frame that would permit observation

of lethal affects and maintenance of appropriate control survivorship. Consequently, the time to mortality in field use of these baits would be expected to be longer.

Chitin synthesis inhibitors influence not only the act of molting, but also physiology (peritrophic matrix, fat body) and biochemical (DNA synthesis) effects associated with chitin synthesis (Retnakaran et al. 1985, Nakagawa and Matsumura 1994, Morales-Ramos et al. 2006). We observed termites that displayed the jackknife pose, a condition attributed to aborted molting (Su and Scheffrahn 1993, Getty et al. 2000). The jackknife pose was recorded in all CSI treatments at low frequency (≤5%) and were comparable to the findings reported by Su and Scheffrahn (1993, 1996) although the jackknife pose was common (35%) in our diflubenzuron replicates (Fig. 3.5). Most of the dead or moribund termites in our test did not display obvious signs of 'molting inhibition' yet we recorded what is termed the body curl pose (Fig. 3.5). This display in a moribund state has not been previously described and could be indicative of chitin synthesis of the peritrophic matrix but further testing would be needed to conclude the effect of CSIs on this structure. An explanation for the body curl posture, prior to death, should be pursued in future research as it may indicate a cause of mortality separate from the molting process.

Baits used for management of subterranean termites can be efficacious under one of two scenarios. The first is that every termite in a targeted population visits the toxicant-laden bait and feeds. This 'all-must-visit' scheme could be successful if termites moved between feeding locations on a ‘regular’ (perhaps daily) basis. The second scenario requires movement of the active ingredient by termites that deliver toxicant to nestmates that do not contact treated bait. There are no field data to corroborate either of the aforementioned scenarios and most of the literature assumes the second scheme. The movement scenario depends on distribution of active

ingredient to termites that never feed on the toxicant-laden bait and by extension the most efficacious bait would be one that is efficiently transferred.

Movement of a food-borne active ingredient within a subterranean termite population is a matter of conjecture although recent observations do provide some insight. Food sharing, traditionally termed "trophallaxis" (Sleigh 2002) involves two different mechanisms of transfer (Whitman and Forschler 2007). Stomodeal and proctodeal exchanges are the most commonly considered food-sharing options employed in interpreting termite bait-toxin transfer data (Sheets et al. 2000, Karr et al. 2004, Haagsma and Rust 2005, King et al. 2005). In short, stomodeal trophallaxis (or autofeeding stomodeal) is a recipient driven process involving donors that are chewing something that could have been obtained from a recently completed stomodeal or proctodeal exchange, or food self-procured by the donor (autofeeding cellulose) (Whitman and Forschler 2007). The model of recipient-driven exchange obviates stomodeal transfer of bait toxins because termites have not been observed chewing food as they move from location to location, and according to Whitman (2007) exchange of regurgitated food is a rare event. If we exclude stomodeal trophallaxis there remains three other ways for a termite to move poison bait from a feeding site. Delivery can be accomplished by dermal contact with contaminated feces and by grooming if the AI is processed to the exterior of the cuticle or if the cuticle is contaminated by contact with the bait or feces. Whitman and Forschler (2007) stated that grooming was the most consistent behavior observed in worker-to-worker interactions and if a bait-borne CSI is excreted on the cuticle, it could be efficiently transferred as demonstrated by the work of Myles (1996). The cuticle/grooming route of CSI transfer should be examined in more detail although Haggsama and Rust (2005) and Sheets et al. (2000) present data indicating that an insufficient amount of radiolabeled CSI is provided to donors by contact with exposed

termites. The possible transfer by coprophagy was also shown to be minimal (Sheets et al. 2000, Haggsama and Rust 2005).

Therefore, we propose that the most likely route of CSI transfer is a proctodeal donation some distance from the toxicant-bait feeding site. The meal taken at a toxin-containing bait station must be provided sometime later, after movement away from the station, yet within the timeframe of 'clearance' or movement of food through the alimentary tract. Clearance or 'half-life' has been described using radiolabeled CSI's as the time an active ingredient remains in the termite body (Sheets et al. 2000). Most of the literature report that CSI "transfer" (and we are assuming the proctodeal route as determined by the experimental design of feeding donors in one arena and placing them with recipients in a separate arena) in termites peaks between 8h to 2 d (Sheets et al. 2000, Karr et al. 2004, Haagsma and Rust 2005, Spomer and Kamble 2006). That 48 hour time frame for movement of a food bolus through the alimentary tract of a subterranean termite matches previous observational data using stained α-cellulose (Forschler 1996) in addition to experiments of radiolabeled food transfer (Suárez and Thorne 2000).

Behavioral responses must also be considered when evaluating the efficacy of a termiticide (Su et al. 1982, Haverty et al. 1989, Forschler and Jenkins 2000). Termites that consume toxicant-laced food must be elicited for a food donation. Distribution of toxin could be compromised by workers that display signs of intoxication that are avoided (not solicited for a donation) and those that are unable to move from the CSI feeding site. In addition, our data showed termites exposed to CSIs were infrequently cannibalized (Fig. 3.4). It is also possible cadavers were more available in CSI treatments and might account for the decrease in cannibalism as compared to our nontreated control (Fig. 3.4). Haagsma and Rust (2005) found transfer of hexaflumuron by cannibalism was efficient only in groups containing donors that

were starved; however further research is needed to elucidate the impact of cannibalism on toxicant transfer. Whitman and Forschler (2007) suggested that observation of the jackknife pose in bioassay is indicative of recognition of unhealthy dead and we never found those individuals cannibalized. However, termites in the jackknife pose were characterized by dorsal side of the thorax swollen (Fig. 3.2A) and might not be able to withstand molting process due to a weakened exoskeleton.

The mortality results from our bioassay (Table 3.1) and the work of Lewis and Power (2006) and Vahabzadeh et al. (2007) indicate that feeding on lufenuron-treated cardboard bait provides transfer comparable to other CSI's. Yet, we observed lufenuron donors that displayed a characteristic stance with antennae held straight forward in a "V" orientation when viewed dorsally. This unusual pose may signify a behaviorally compromised termite because these same donors did not respond by increased movement, as did the controls (lifting the Petri dish lid). We propose that lufenuron would not be effectively transferred in the field because the behavior of the potential donors is compromised.

In conclusion, despite the fact that the dose required to achieve mortality has not been determined for any CSI (Sheets 2000), the commercial bait formulations we tested transferred in an efficient manner within the confines of a Petri dish and suggests a low (ng AI per termite) dose/mortality relationship. Very high efficiency of transfer is indicated by our observation of >90% mortality when 19 non-exposed nestmates were confined with a single termite that had been exposed to CSI bait for 7 days. The actual mode(s) of transfer remains unresolved but sufficient evidence is provided to suggest the main route is proctodeal donation. The reduced efficiency of diflubenzuron at the lowest D:R's tested may be a result of the lower concentration of that CSI bait (0.25%) compared to the three baits with a 0.5% concentration. The lowest

concentration of CSI in the baits we tested was lufenuron at 0.15% and it provided equivalent mortality with all but the lowest D:R (1:19); yet the behavioral observations suggest that this CSI will not be effectively transferred under field conditions. This is supported by observations of movement of lufenuron by donors and the assumption that movement away from the bait site is a critical component of termite bait field efficacy.

3.6 References

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Table 3.1. Lethal time estimates (LT_{50} and LT_{90}) (±95% CI) by chitin synthesis inhibitor and donor to recipient ratios along with corresponding regression slopes from *R. flavipes*.

Trmt[1]	D:R[2]	LT_{50} (95% CI) [3, 4]		LT_{90} (95% CI) [3, 4]		Slope ± SE
CON	*	253 (218-304)		456 (390-552)		0.20 ± 0.02
EXP	20:00	24 (22-25)	A	42 (40-45)	A	2.16 ± 0.13
	15:05	24 (22-25)	A	43 (40-48)	A	2.04 ± 0.17
	10:10	23 (22-24)	A	42 (40-44)	A	2.11 ± 0.10
	5:15	27 (26-29)	B	46 (43-50)	A	2.13 ± 0.13
	1:19	25 (24-27)	AB	44 (41-46)	A	2.21 ± 0.12
DFB	20:00	24 (22-26)	A	43 (40-46)	A	2.13 ± 0.16
	15:05	27 (25-28)	A	45 (42-48)	A	2.20 ± 0.12
	10:10	28 (27-29)	A	48 (46-50)	A	1.99 ± 0.08
	5:15	54 (47-68)	B	97 (80-129)	B	0.94 ± 0.15
	1:19	67 (59-78)	B	121 (104-148)	B	0.73 ± 0.08
HEX	20:00	23 (22-24)	A	40 (39-42)	A	2.28 ± 0.11
	15:05	22 (21-23)	A	39 (38-41)	A	2.33 ± 0.10
	10:10	26 (25-27)	B	43 (41-45)	AB	2.31 ± 0.09
	5:15	26 (25-27)	B	43 (41-46)	AB	2.29 ± 0.11
	1:19	27 (26-28)	B	45 (43-47)	B	2.28 ± 0.08
NFM	20:00	25 (23-27)	A	43 (40-46)	A	2.23 ± 0.15
	15:05	25 (23-27)	A	44 (41-48)	A	2.07 ± 0.14
	10:10	26 (25-27)	A	44 (42-45)	A	2.25 ± 0.08
	5:15	29 (27-31)	A	48 (44-53)	A	2.09 ± 0.16
	1:19	29 (27-30)	A	47 (44-50)	A	2.18 ± 0.12
LUF	20:00	17 (13-19)	A	39 (36-44)	A	1.76 ± 0.19
	15:05	21 (19-23)	A	39 (37-42)	A	2.18 ± 0.16
	10:10	25 (24-26)	B	44 (42-47)	A	2.10 ± 0.10
	5:15	26 (24-27)	B	44 (41-47)	A	2.21 ± 0.14
	1:19	32 (31-34)	C	53 (50-57)	B	1.91 ± 0.11

[1] Trmt = treatments were exposed to termites for 7 days and include: CON = control (α cellulose), EXP = experimental CSI, DFB = diflubenzuron, HEX = hexaflumuron, NFM = noviflumuron, LUF = lufenuron. Mortality data were taken daily for 42 days after initial exposure.

[2] D:R = donors to recipients ratio.

[3] LT = lethal time by day for 50% or 90% mortality.

[4] CI = confidence intervals followed by the same upper case letter indicate intervals overlap and cannot be rejected within a treatment.

* Percent mortality (± SD) between ratios are not significantly different as determined by Tukey-Kramer HSD (F=0.2213; p=0.639).

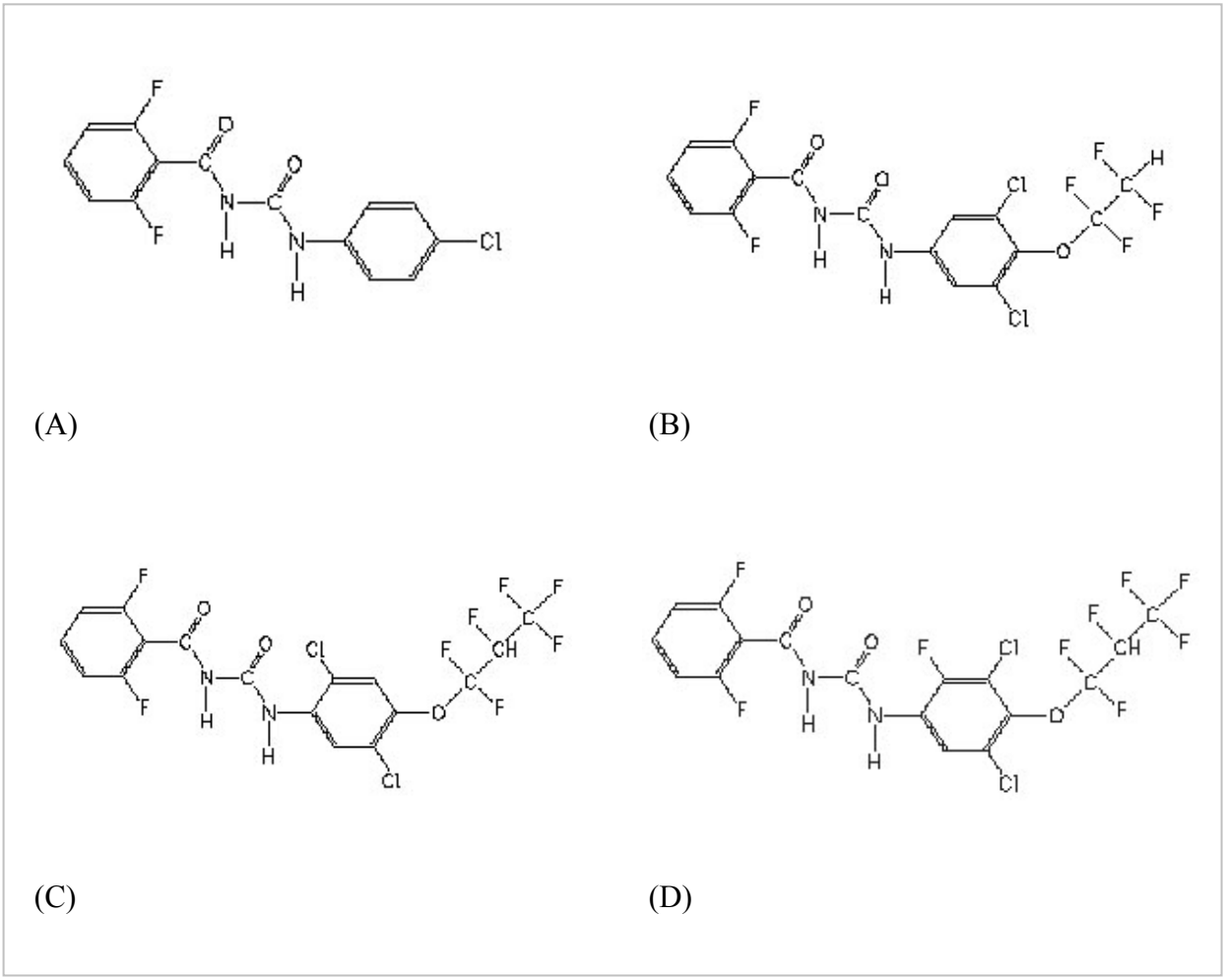

Figure 3.1. Chemical structure of benzoylphenyl urea insect growth regulators. (A) Diflubenzuron. (B) Hexaflumuron. (C) Lufenuron. (D) Noviflumuron.

(A)

(B)

Figure 3.2. Physical deformities observed in termites exposed to chitin synthesis inhibitors. (A) Jackknife pose. (B) Curled body.

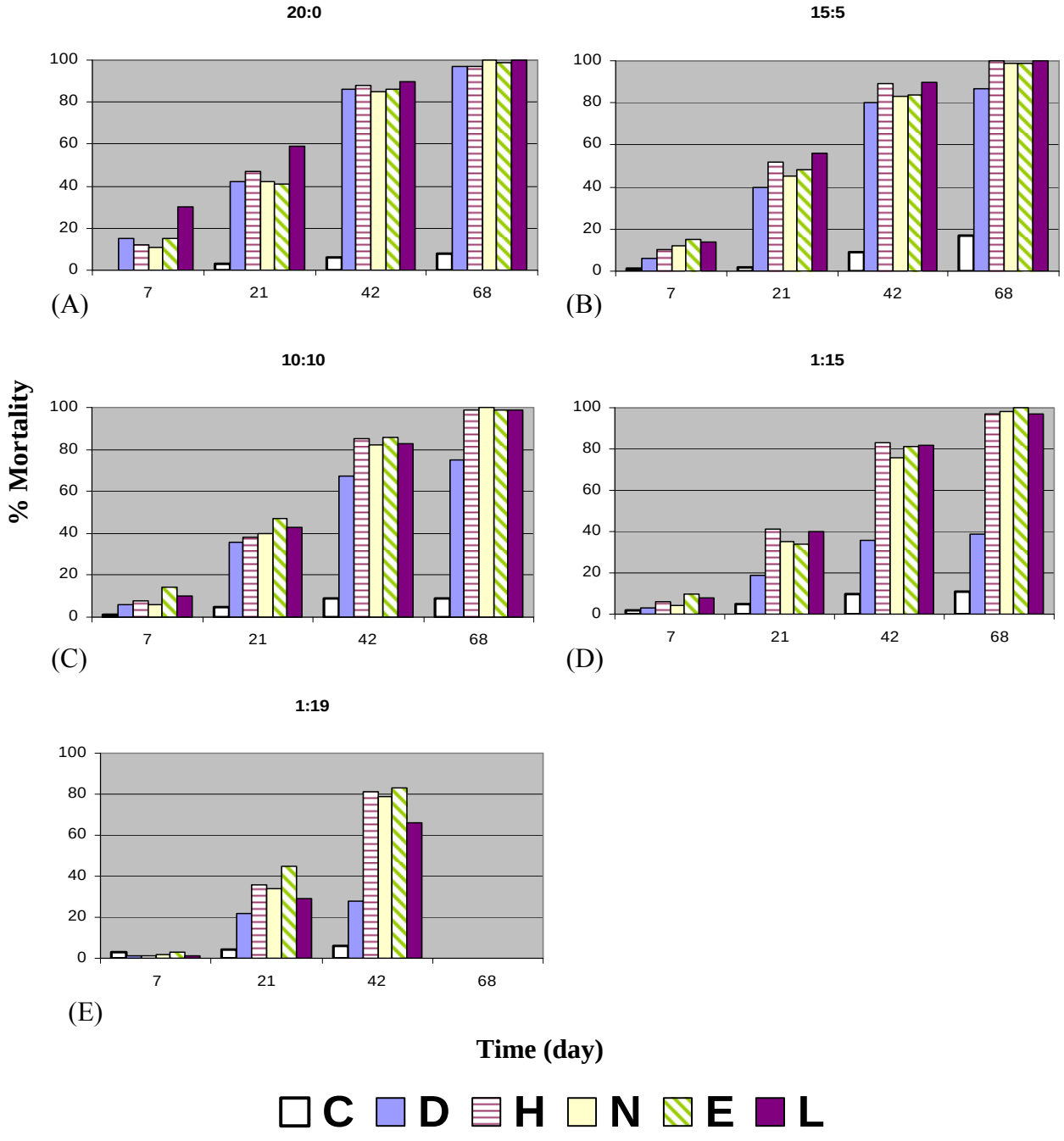

Figure 3.3A-E. *Reticulitermes flavipes* mortality over time (7, 21, 42, and 68 day) exposed to chitin synthesis inhibitors (CSIs) at various donor to recipient ratios. CSI exposures include C=control, D=diflubenzuron, H=hexaflumuron, N=noviflumuron, E=experiment CSI, and L=lufenuron. Ratio of donors exposed to CSI to recipients (D:R). (A) 20:0, (B) 15:5, (C) 10:10, (D) 5:15, and (E) 1:19.

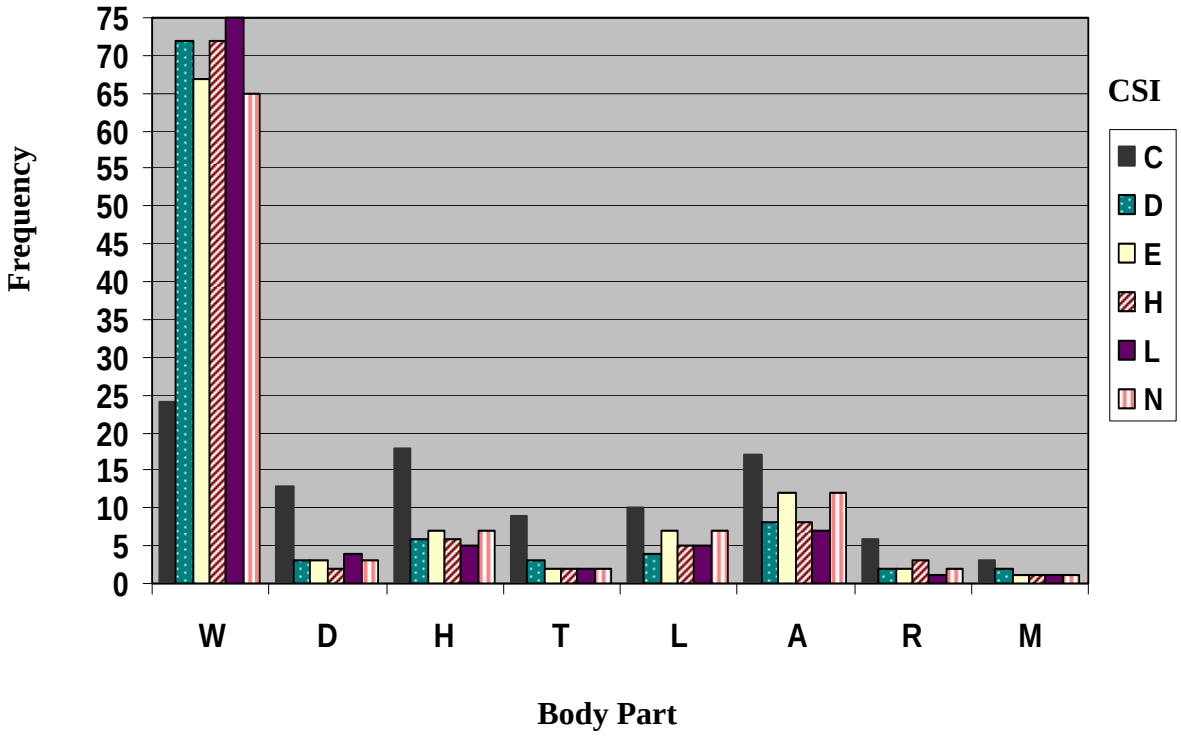

Figure 3.4. Evidence of cannibalism observed in *R. flavipes* post-exposure to chitin synthesis inhibitors (CSIs) at various donor to recipient ratios. CSI exposures include non-active C=control, D=diflubenzuron, H=hexaflumuron, N=noviflumuron, E=experiment CSI, and L=lufenuron. Observation of the dead and signs of cannibalistic acts noted over 68 day period are; W=whole body intact, or appendages missing; D=abdomen; H=head; T=thorax; L=legs; A=antennae; R=body buried; or M=whole body missing.

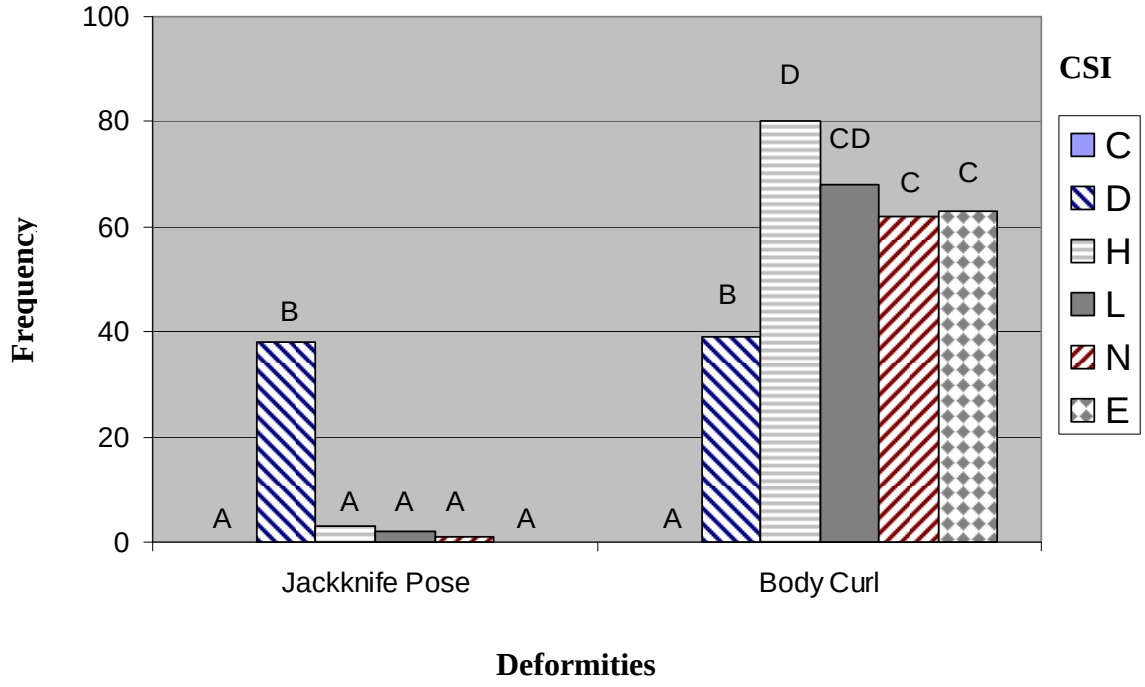

Figure 3.5. Frequency of dead or moribund *R. flavipes* exhibiting physical deformities following exposure to chitin synthesis inhibitors (CSIs) over a 68-day period. CSI exposures include non-active C=control, D=diflubenzuron, H=hexaflumuron, L=lufenuron, N=noviflumuron, and E=experiment CSI. Physical deformities were jackknife pose and body curl. Different letters indicate significant differences ($p<0.05$) to bait treatment by deformity.

CHAPTER 4

TRANSFER OF CSI TOXICANTS BY CANNIBALISM DEPENDS ON TERMITE BEHAVIOR

4.1 Introduction

The contribution cannibalism plays in toxicant transfer is not known in termites. Cannibalism is defined as intraspecific predation, including cadavers, and has been recorded from several animal groups including; planaria, protists, rotifers, snails, centipedes, mites, copepods, fish, birds, mammals and insects (Fox 1975, Polis 1981). This behavior occurs in both artificial and natural environments as a survival strategy caused by the availability of food, population density, stress, and certain mating systems (Fox 1975, Elgar and Crespi 1992). Cannibalism is a common survival strategy found in insects from enclosed feeding niches, such as seeds, fruits, stored products, and wood (Simpson et al. 2006). Previous termite studies have shown limiting food resources will change their behavior, biology, morphology, and physiology (Esenther 1969, Grosovsky and Margulis 1982, Badertscher et al. 1983, Su and LaFage 1986, Gulmahamad 2002, Song et al. 2006). These eusocial insects also cannibalize nestmates as a defensive strategy to reduce disease transmission (Kramm et al. 1982, Jones et al. 1996, Myles 2002a).

Subterranean termites are an important structural pest in the urban environment with preventative and control measures estimated at $2 billion annually in the United States alone (Jones 2003). Food-borne toxicants containing chitin synthesis inhibiting (CSI) bait formulations are one method used to manage termite populations. These chemistries interfere with an insect's physical development and cuticle sclerotization during the molting process

(Cohen 1987). Cannibalism is frequently cited in the literature as a mechanism of toxicant transfer in termites; however, empirical evidence is rare and incomplete (Ferster et al. 2001, Thorne and Breisch 2001, Hu et al. 2005).

My objective was to investigate the contribution cannibalism plays in toxicant transfer by allowing termites to ingest dead nestmates previously exposed to a CSI. Acceptance of treated cadavers should also be considered when evaluating a food-borne toxicants. We compared five commercially available CSIs to determine the effect of cannibalism on termite mortality. CSIs included diflubenzuron 0.25% (Whitmire Micro-Gen, St. Louis, MO), hexaflumuron 0.50% (Dow AgroSciences, Indianapolis, IN), noviflumuron 0.50% (Dow AgroSciences, Indianapolis, IN), lufenuron -treated corrugated cardboard (0.15%; Syngenta Corporation, Greensboro, NC) and an experimental CSI (Whitmire Micro-Gen, St. Louis, MO). Observed behavioral conditions included signs of toxicity (molting inhibition, ataxia, and the condition of the dead including absence of body parts) were recorded. We hypothesized cannibalism of nestmates would decrease estimated lethal time. We predicted that because these toxicants are provided at a nonrepellent concentration, then termite cadavers previously exposed to CSIs would be cannibalized and transfer a lethal dose. Given the effectiveness of CSIs, then those individuals confined with dead nestmates should potentially transfer toxicant by cannibalism and therefore exhibit higher mortality.

4.2 Material and Methods

Insects and Chemicals. Three populations of *Reticulitermes flavipes* (Kollar) were brought back into the laboratory intact in their food source in Whitehall Forest, Clarke Co., GA. Termites were collected using moistened corrugated cardboard, and once collected, they were

placed into plastic containers (7-3/8 x 3-11/16 x 3) with weathered pine wood slats in complete darkness inside an environmental chamber (27° C, ≥90% RH) until used in bioassays (Forschler and Townsend 1996).

Approximately 200 worker (fourth instar or older) termites used as donors were placed into a Petri dish (100X25 mm) with 20 grams of sand, 3.2-ml of distilled water and a known weight (6.5 ± 1.5g) of commercially available α-cellulose tablet containing either no CSI (control), diflubenzuron 0.25% (Whitmire Micro-Gen, St. Louis, MO), hexaflumuron 0.50% (Dow AgroSciences, Indianapolis, IN), noviflumuron 0.50% (Dow AgroSciences, Indianapolis, IN), lufenuron -treated corrugated cardboard (1.5 ± 0.3 g at 0.15%; Syngenta Corporation, Greensboro, NC), or a noncommercial experimental CSI (Whitmire Micro-Gen, St. Louis, MO), for 7 d. Each CSI treatment had a corresponding non-treated control set up just as described for the control donors, with α-cellulose. All termites were observed prior to their placement into a Petri dish at 60X magnification to verify body parts were not missing. For inclusion in the bioassay, termites only were included if they appeared to have eaten as indicated by their distended abdomen.

Transfer by Cannibalism. Donor termites were placed into a plastic Petri dish (65X15 mm diameter) containing moistened filter paper (Whatman #1, 55 mm diameter) along with termites from the same population (recipient termites) to provide a donor to recipient ratio of 1:1 for a total of 20 workers. Donor termites were marked with DecoColor Paint Marker (Uchida American DecoColor Paint marker) and recipients treated as described above but exposed to untreated cellulose (control). Included with every treatment replicate was a corresponding control replicate that was set to a limit of <20% mortality in the corresponding control as the benchmark for including the treatment data into the dataset used in my analysis.

Termite cadavers were treated one of two ways, either both the dead and moribund were removed (Rd) on a daily basis or they were left (Ld) confined with living termites in the Petri dish and cumulative mortality determined for 49 d. There were 5 replicates per termite population. To remove a dead or moribund termite, clean forceps were used to grasp the head so as no bodily fluids containing possible le toxicant could potentially be exposed to other termites. Body parts missing were verified under a stereomicroscope at 60X magnification.

Cannibalized Body Parts. Cannibalistic acts were recorded, and any missing termites or missing appendages were assumed cannibalized, including missing legs, antennae, abdomen, head, thorax, and presence or absence of the whole cadaver. We also collected information about "jackknife" pose as noted previously (Su and Scheffrahn 1993), indicative of molting inhibition, as well as a physical deformities we termed 'body curl' that did not display signs of recent molting.

Consumption of Introduced Cadavers. Previous studies have prepared termite corpses by freezing them (Haagsma and Rust 2005, Song et al. 2006). In my initial study, freeze-killed termites began to decay after 3-4 days and mortality in the nontreated control was high (Fig. 4.4). Two techniques to kill termites after exposure to a CSI were compared to see if the method contributed to avoidance behavior. Termites were either freeze-killed or dried-killed following 7-day exposure to the CSIs, noviflumuron or lufenuron. Individuals that appeared to have fed on bait were chosen for preparation of cadavers by placing them individually in 96-well plate (Falcon, Becton Dickinson and Co., Lincoln Park, NJ) and either frozen at -18° C, or allowed to desiccate at room temperature in a window.

Seven killed termites were added, one per day to a Petri dish with 19 recipient termites or non-exposed nestmates. Termite behavior was noted daily for a total of 24 days. Any burial

piles were carefully examined for the absence of specific party parts. Termite cadavers were treated one of two ways, as stated above, with either the dead removed (Rd) or the dead left (Ld) and examined on a daily bases. Therefore, after 8 days, the Rd still maintained a total of 19 termites while the Ld had 26. This was replicated 5 times per treatment and termite population.

Data collected included termite mortality, the absence of body parts from cadavers, and behavior of living termites. Because of daily observation, I could note any missing body parts during the burial of cadavers. Termite behavior was assigned a subjective rating from 10 to 3≥ based on their response when the lid of the Petri dish was removed and ranged from a quick response followed by actively running around the Petri dish to not responding at all (Table 4.2). Any response equal to or less than 3 could not be discerned as living or dead, therefore this was grouped together (Table 4.2).

Statistical Analysis. Mortality data were adjusted using Abbott's formula (1925) and compared using SAS-JMP (version 7.0) statistical software (SAS Institute, Inc., Cary, NC) by treatment of the dead, CSI, and time. The effects of termite origin were controlled for in the statistical model. Termite mortality and cannibalism were evaluated by analysis of variance (ANOVA) and Tukey-Kramer honestly significant difference (HSD) test for multiple mean comparisons ($\alpha = 0.05$). Data were also subjected to probit regression to estimate 50% and 90% lethal time (LT) estimates. If confidence intervals (CI) ($\alpha = 0.05$) of LT estimates did not overlap, they were not significantly different.

4.3 Results

Transfer by Cannibalism. Termite mortality tended to be increase when confined with cadavers (Ld) previously exposed to diflubenzuron, hexaflumuron, and lufenuron (Fig. 4.1;

df=23; F=143.88; p<0.0001). Termite mortality increased from 45% when dead were removed to 57% when dead were left by 21 days (Fig. 4.1; df=3, 26; F=3.75; p=0.0231). Termite mortality for individuals confined with cadavers had similar results from hexaflumuron by day 21 (68% vs. 53%; df=3, 26; F=3.93; p=0.0195) and 14% greater by 28 days (87% 21 vs. 75%; df=3, 26; F=5.49; p=0.0047). Lufenuron had twice the termite mortality when confined with cadavers by days 7 (35%; df=3, 26; F=8.15; p=0.0005) and 14 (67%; df=3, 26; F=33.74; p<0.0001) (Fig. 4.1). We did not find a difference between treatment of the dead from termites exposed to noviflumuron or the experimental CSI (Fig. 4.1).

The LT_{50} ranged between 20-23 days, following the initial 7 day exposure to noviflumuron or the experimental CSI, regardless of treatment (Rd and Ld) (Table 4.1). There were similar findings (termite LT_{50} of ~3 wks) in hexaflumuron and lufenuron when the dead were removed however, estimates were significantly shorter (18 d and 7 d, respectively) if dead left (Table 4.1). Termites exposed to diflubenzuron with the dead left had LT_{50} estimates of 22 days while when the dead removed was 28 days (Table 4.1).

LT_{90} estimates were approximately 6-7 wks for all CSIs and did not differ by treatment of the dead (Table 4.1).

Cannibalized Body Parts. When termite cadavers were removed daily from the nontreated control, I did not find a difference in cadavers having missing body parts, buried, or left entirely intact (Fig. 4.2A; df=7; F=1.91; p=0.1860). In contrast, termites exposed to the CSIs, diflubenzuron, experimental CSI, hexaflumuron, and noviflumuron had approximately 90% (88-93%) of cadavers not cannibalized (Fig. 4.2A; F=13.44; df=5, 24; p<0.0001). Similar findings were recorded from lufenuron-exposed termites but only 78 ± 20% of the dead remained uncannibalized (Fig. 4.2A).

Termites from the nontreated controls confined with cadavers any dead individual cannibalized and buried (Fig. 4.2B). Termites exposed to CSIs had a significant number of cadavers not cannibalized over 49 days (Fig. 4.2B; $F=25.42$; $df=5, 24$; $p<0.0001$). Approximately half of the dead termites exposed to hexaflumuron (64 ± 11%), noviflumuron (67 ± 8%), and lufenuron (68 ± 23%) where left intact with no signs of missing body parts (Fig. 4.2b). Only about 45 ± 8% of the dead in the experimental CSI treatment were not cannibalized and 20 ± 9% in diflubenzuron (Fig. 4.2B). We frequently noted buried termites with missing appendages (legs and antennae seemed most frequent) under filter paper and/or frass, however when mortality increased to at least 50%, it was hard to distinguish individual termites in a burial pile. Therefore, percent-cannibalized appendages could be underestimated in Ld treatment of CSI tablets.

Two physical deformities were observed in moribund termites exposed to CSIs termed "jackknife" pose and "body curl" (Fig. 4.3). Cadavers removed that had a jackknife pose were only seen from termites in diflubenzuron treatment (23 ± 14%) (Fig. 4.3). We also observed termites with a physical deformity we describe as "body curl", characterized by the thoracic terga constricting to form a U shape and an inability to move, only seen in moribund individuals. "Body curl" in moribund was seen from all CSI treatments and ranged from 56-89% of the individuals removed (Fig. 4.3).

Consumption of Introduced Cadavers. The initial experiment of adding only freeze-killed termites had high mortality (Rd= 13 ± 8% and Ld= 25 ± 16%) by day 12 (Fig. 4.4). This provided important information on how cadavers are cared for by termites in non-treated controls. There were a large number of cadavers missing antennae and legs due in large to head

or thorax presumably consumed (Fig. 4.4). Untreated termite cadavers were always cannibalized and or buried (100%), regardless of time left (Rd and Ld) in a Petri dish (Fig. 4.4).

Efficiency of CSI transfer by cannibalism was not effected by freeze-killed or dried-killed technique (df=1; F=3.37; p=0.0684), therefore further analyses based on pooled data. The LT_{50} data ranged between 23-30 days for termites in noviflumuron and lufenuron, regardless of treatment (Rd and Ld) (Table 4.1). LT_{90} estimates were approximately 7 wks (35-59) for all CSIs and did not differ by treatment of the dead (Table 4.1). Consumption of introduced cadavers from noviflumuron and lufenuron had significant mortality in termites by treatment (df=2; F=105.47; p<0.0001), and time (df=3; F=3.55; p<0.0001) (Fig. 4.5).

All termite cadavers were cannibalized from the nontreated control and a large number from CSI treatments (82% of cadavers from noviflumuron and 74% from lufenuron (Fig. 4.6). There were also a large number of cadavers buried from noviflumuron (76%) and lufenuron (83%) treatments (Fig. 4.6; df=2, 5; F=4.48; p=0.0159).

A termites' ability to remain "active" was impaired by killed termites that had been exposed to a CSI (Fig. 4.7). By day 6, termites already were seen having difficulty in responding to stimuli (Petri dish lid removed) (Fig. 4.7; df=5; F=75.69; p<0.0001). Individuals exposed to lufenuron also exhibited signs of ataxia and would be unable to forage.

4.4 Discussion

Cannibalism by termites is an important mechanism for the acquisition of nitrogen for protein synthesis (Moore 1969, Lenz 1994, Shellman-Reeve 1994, Curtis and Waller 1997). It has been proposed termites will cannibalize nestmates when they are starved (La Fage and Nutting 1978, Song et al. 2006) but these studies do not determine age prior to use in bioassays. Mortality is a natural and ultimate ending for all living organisms. I observed a high frequency

of cadavers cannibalized by termites in untreated control, and there was no impact on mortality (Figs. 4.1-4.2, 4.6-4.7). These findings agree with Song et al. (2006), that cannibalism of appendages is common but cannibalism of an entire termite was rare. Termites within the experimental CSI set up had plenty of food provided and did not show signs of starvation. For inclusion in the bioassay, termites, whether from the nontreated control or treated termites, were only included if they appeared to have eaten as indicated by their distended abdomen.

There are a number of publications referring to the transferability of toxicant by cannibalism (Su and Scheffrahn 1993, Sheets et al. 2000, Valles and Woodson 2002), but empirical evidence is scanty. Mechanisms for toxicant transfer among termites takes advantage of food exchange between nestmates either by a recipient requesting food from another's mouth (allofeeding stomodeal) or hindgut exudes from their anus (allofeeding proctodeal) (Whitman and Forschler 2007). CSIs are presented to termites as toxicant-laced food at a nonrepellent and slow acting concentration (reviewed in Su and Scheffrahn 1998, Su 2003), and under these circumstances, we could expect cannibalistic behaviors to CSI treated individuals based on normal and non-intoxicated behavior. Cannibalism in this situation could potentially expose naïve nestmates to a lethal dose and potentially transmit to other nestmates before the onset of acute toxicity or avoidance behavior. Mechanism minimizing exposure to pathogens include recognition, avoidance, and burial of infected individual (Jackson and Hart 2008), and any behaviors associated could explain discrepancies found in field studies with CSI baiting systems.

A termite's ability to follow a pheromone trail following intoxication might be a factor in horizontal toxicant transfer if these individuals are "marked" as sick and consequently avoided and shunned. It was evident termites were able to detect individuals previously exposed to CSIs and isolate them by entombment (Figs. 4.2A and B). Rosengaus et al. (1999) observed fungal

exposed termites using mechanoreceptive communication system by striking the substrate, and then, subsequently they were avoided by nonexposed nestmates. Another behavior I noted were individuals that appeared 'sick' and still alive would occasionally back themselves against burial piles prior to their mortality. Further studies looking at this type of altruistic behavior

Subterranean termites are constantly exposed to microbes because of their life history and from their environment. Myles (2002b) found fungal exposed termites displaying unusual foraging behavior and were attacked. These 'sick' termites were immobilized with bite wounds to their legs, later defecated upon, and buried alive with bits of chewed filter paper incorporated in the burial pile (Myles 2002b). Termite sanitation behavior also inhibits transmission of fungi and other pathogens by avoidance, entombment, and cannibalism (Boucias et al. 1996). Cannibalism is an important mechanism for waste disposal and sanitation (in the defense against disease) (Wilson 1971, Rosengaus and Traniello 2001, Myles 2002a). Termites will also show signs of aggression, like biting appendages off from those that appear intoxicated or show signs of ataxia, sluggishness, or other unusual foraging behavior (Weesner 1956, Smythe and Carter 1970, Mannesmann 1973, Grosovsky and Margulis 1982, Su and La Fage 1986, Rosengaus and Traniello 2001, Myles 2002a, Osbrink and Lax 2002).

Dead or dying termites can have a repellent effect on remaining termite workers as seen by Su et al. (1982) and Evans et al. (1998). CSI treated termites had to be alive to elicit an avoidance response (Figs. 4.2B and 4.6) and could be explained by behavioral responses to contact. Burying the dead is one way to avoid disease transmission and perhaps these burial piles covered in frass and pieces of filter paper served as a antimicrobial agent. A microbial comparison between cadavers in a burial pile versus those not buried could prove insightful into some of these differences noted.

The results of this study provide information on transmission efficiency of CSIs by cannibalism. This study also raises questions of how termites are recognized as "sick" and as a result, being avoided. Avoidance to sick termites might be just behavioral observations or other factors can be involved. Further research looking at the efficacy of IGR termiticides is needed to see how termites behave in field setting. This information should help with future termite bait management strategies.

4.5 References

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Table 4.1. Lethal time estimates (LT_{50} and LT_{90}) (±95% CI) by treatment of cadavers exposed to chitin synthesis inhibitors and corresponding regression slopes from *R. flavipes*.

CSI[1]	Cadavers[2]	LT_{50} (95% CI)[3, 4]		LT_{90} (95% CI)[3, 4]		Slope ± SE
C	Rd	328 (219-725)	a	603 (394-1364)	a	0.15±0.04
	Ld	190 (153-255)	a	337 (268-462)	a	0.27±0.04
D	Rd	28 (26-30)	b	54 (49-60)	b	1.51±0.14
	Ld	22 (19-24)	cde	47 (43-52)	b	1.66±0.16
E	Rd	23 (21-24)	c	42 (40-44)	b	2.09±0.19
	Ld	21 (20-23)	c	40 (38-41)	b	2.18±0.10
H	Rd	22 (21-23)	c	39 (38-41)	b	2.24±0.09
	Ld	18 (17-19)	e	37 (35-39)	b	2.16±0.11
N	Rd	22 (21-23)	cd	39 (38-41)	b	2.31±0.09
	Ld	22 (20-23)	cd	38 (37-40)	b	2.40±0.11
L	Rd	20 (18-21)	de	41 (39-43)	b	1.90±0.10
	Ld	7 (1-11)	f	36 (33-40)	b	1.36±0.13

1 CSI = chitin synthesis inhibitors were exposed to termites for 7 days and include C= control (α-cellulose), D = diflubenzuron, E = experimental CSI, H = hexaflumuron, N = noviflumuron, and L = lufenuron. Mortality data were taken daily for 49 days after initial exposure.

2 Cadaver treatment; Ld = leave dead or Rd = remove dead daily.

3 LT = lethal time by day for 50% or 90% mortality.

4 CI = confidence intervals followed by the same lower case letter indicate intervals overlap and cannot be rejected by treatment.

Table 4.2. Behavior description (10-1) based on removing Petri dish lid and observing *R. flavipes* response.

Behavioral score	Description of behaviors
10	"Alarmed" quick response and actively running around
9	Slight stall but still "alarmed"
8	Second stall and running around
7	Few second stall and only moderate "alarm"
6	Can move but are not "alarmed"
5	Move antennae or legs and can barely walk around
4	Move antennae or legs but do not walk around
3≤	Not moving and cannot discern between living and dead

Table 4.3. Lethal time estimates (LT_{50} and LT_{90}) (±95% CI) of introduced cadavers exposed to chitin synthesis inhibitors and corresponding regression slopes from *R. flavipes*.

CSI[1]	Cadavers[2]	LT_{50} (95% CI) [3, 4]		LT_{90} (95% CI) [3, 4]		Slope ± SE
C	Rd	136 (81-746)	a	241 (138-1381)	a	0.38±0.15
	Ld	702 (219-1225)	a	1262 (394-2164)	a	0.10±0.1
N	Rd	29 (26-35)	b	48 (42-59)	b	2.16±0.25
	Ld	26 (23-30)	b	42 (36-50)	b	2.56±0.29
L	Rd	25 (23-27)	b	39 (35-45)	b	2.76±0.24
	Ld	26 (24-30)	b	42 (37-48)	b	2.56±0.29

1 CSI = chitin synthesis inhibitors exposed to termites for 7 days include C= control (α-cellulose), N = noviflumuron, and L = lufenuron. Mortality data were taken daily for 24 days after initial exposure.
2 Cadaver treatment; Ld = leave dead or Rd = remove dead daily.
3 LT = lethal time by day for 50% or 90% mortality.
4 CI = confidence intervals followed by the same locwer case letter indicate intervals overlap and cannot be rejected by treatment.

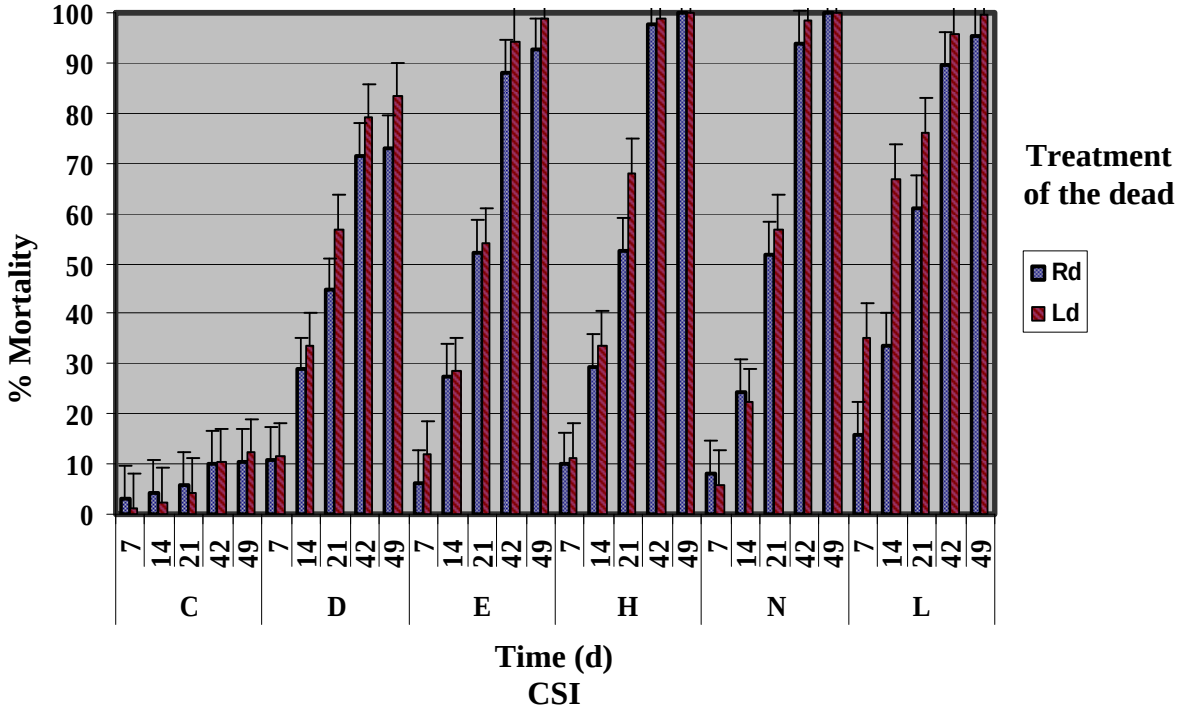

Figure 4.1. *Reticulitermes flavipes* mortality following chitin synthesis inhibitors (CSI) exposure of either having the dead removed (Rd) or left (Ld) in confinement with living nestmates. C= control (α-cellulose), D=diflubenzuron, E=experimental CSI CSI, H=hexaflumuron, N=noviflumuron, and L=lufenuron.

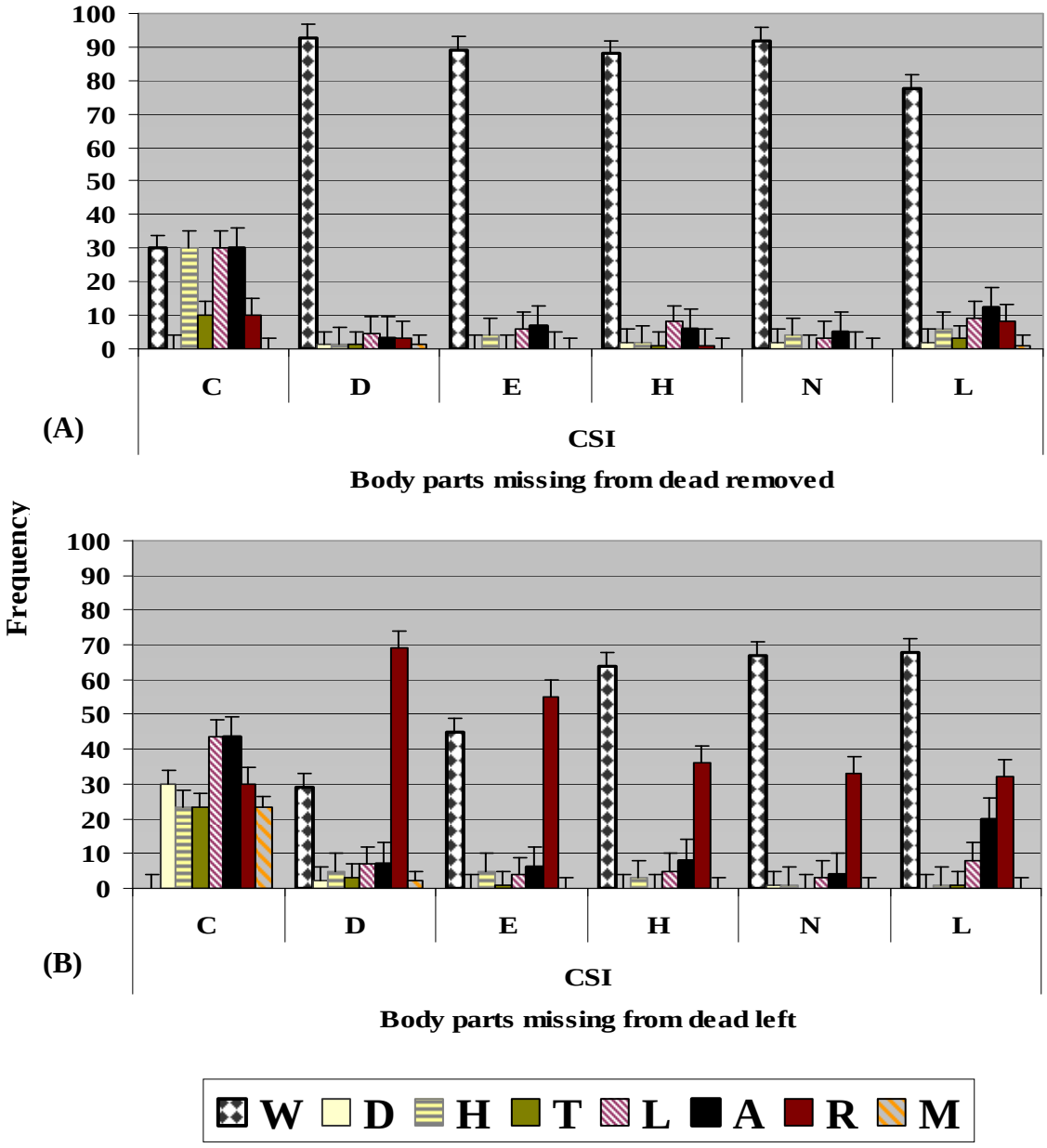

Figure 4.2 A-B. Cannibalistic behaviors observed in *R. flavipes* post-exposure to chitin synthesis inhibitors (CSI) either having cadavers removed or left in confinement. (A) Dead remove daily. (B) Dead left. CSI = chitin synthesis inhibitors included C= control (α-cellulose), D = diflubenzuron, E = experimental CSI, H = hexaflumuron N = noviflumuron, and L = lufenuron. Observation of the dead and signs of cannibalistic acts noted over 49-day period were; W=whole body intact and appears uncannibalized, or appendages missing; D=abdomen; H=head; T=thorax; L=legs; A=antennae; R=body buried; or M=whole body missing.

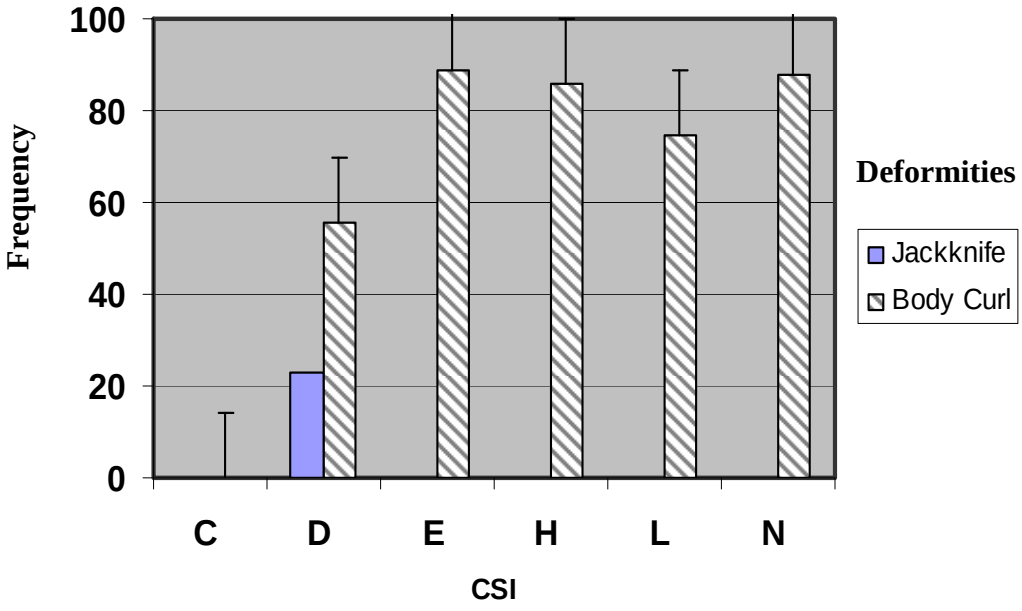

Figure 4.3. Frequency of *R. flavipes* exhibiting physical deformities as occurred in dead individuals removed daily over 49-day post chitin synthesis inhibitors (CSIs) exposure. CSIs include C= control (α-cellulose), D = diflubenzuron, E = experimental CSI, H = hexaflumuron, L = lufenuron, and N = noviflumuron.

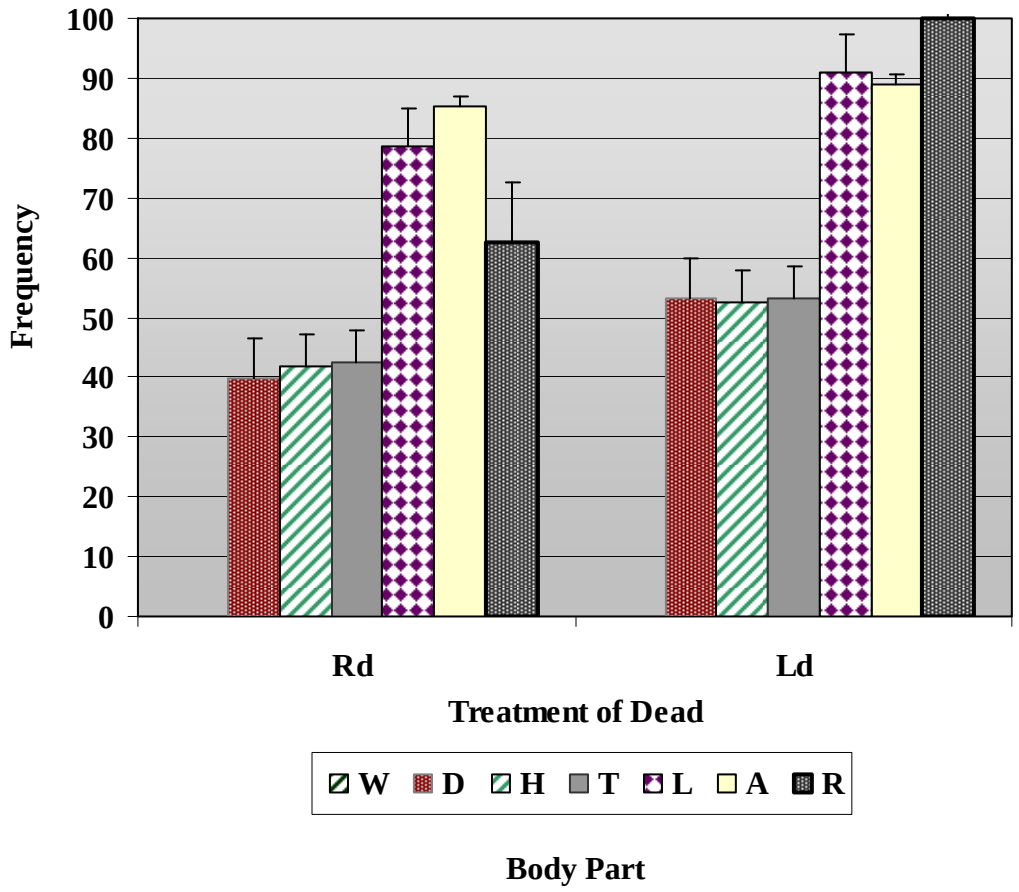

Figure 4.4. Cannibalistic behaviors observed in *R. flavipes* post-exposure to cellulose with either the dead removed (Rd) or dead left (Ld) with living nestmates. Observation of cadavers as noted over a 12 day period are; W=whole body intact, or cannibalistic acts as noted by missing the following appendages; D=abdomen; H=head; T=thorax; L=legs; A=antennae; M=whole body missing or R=body buried.

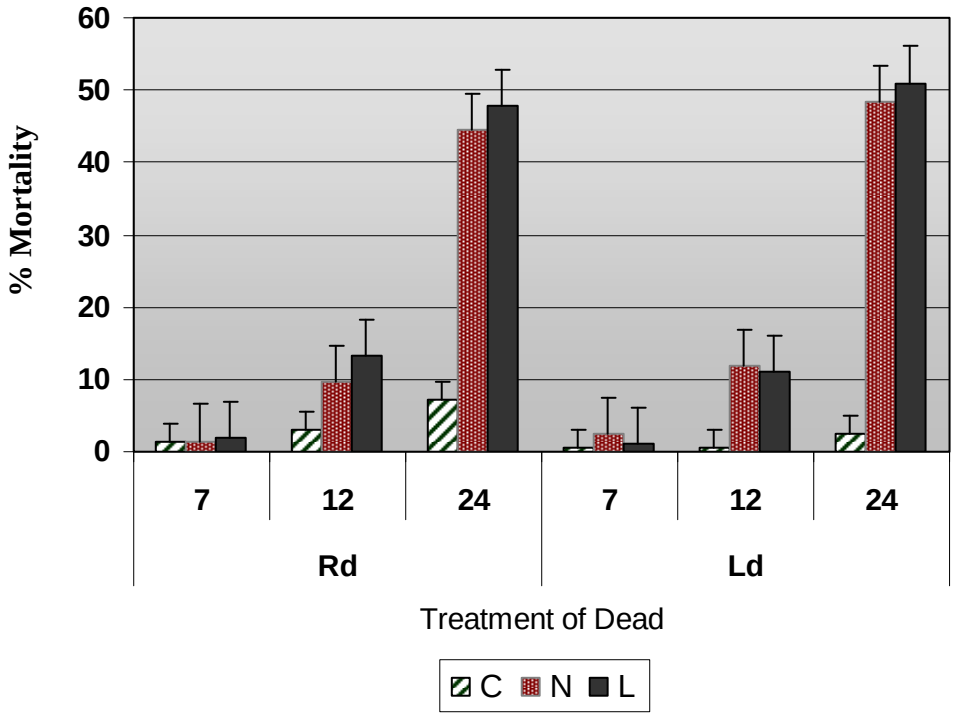

Figure 4.5. *Reticulitermes flavipes* mortality following chitin synthesis inhibitors (CSI) exposure of either having the dead removed (Rd) or left (Ld) in confinement with living nestmates. C= control (α-cellulose), N=noviflumuron, and L=lufenuron.

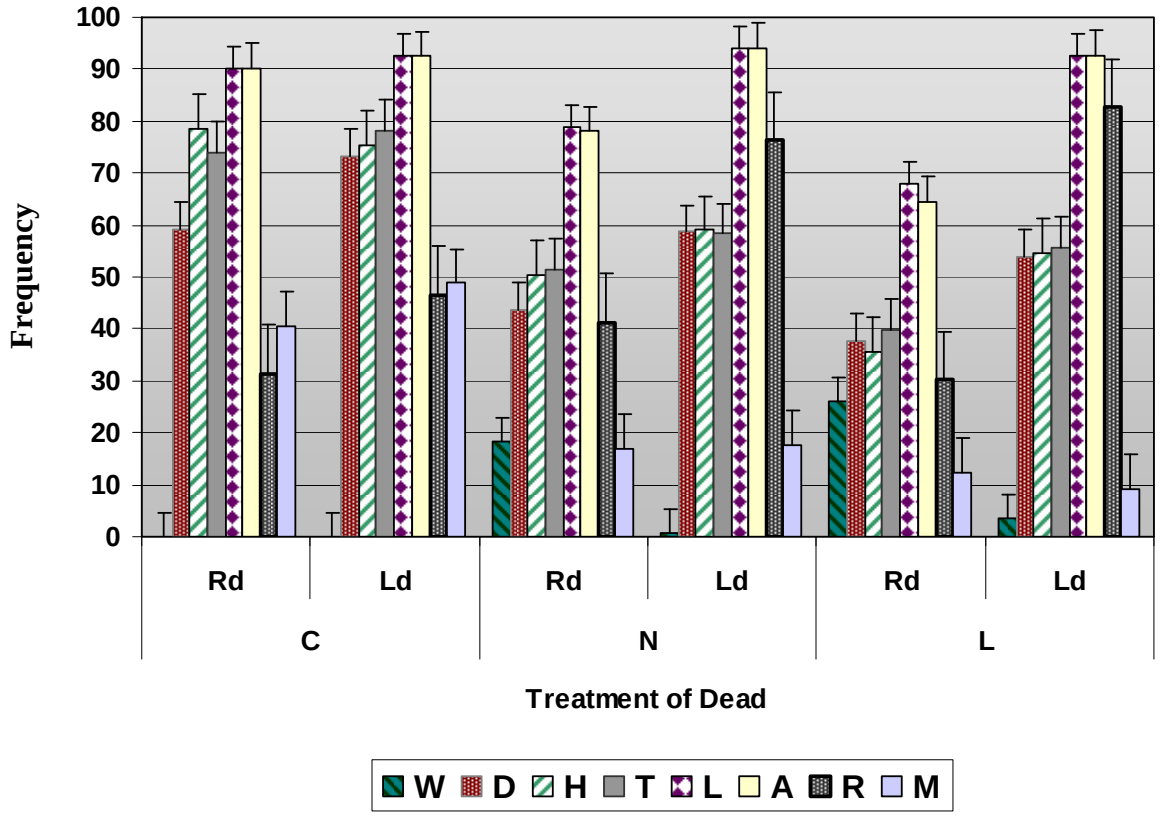

Figure 4.6. Cannibalistic behaviors observed in *R. flavipes* post-exposure to chitin synthesis inhibitors (CSI) either in having the dead removed (Rd) or left (Ld) in confinement with cadavers. C= control (α-cellulose), CSI = chitin synthesis inhibitors included N = noviflumuron, and L = lufenuron. Observation of the dead and signs of cannibalistic acts noted over 24 day period are; W=whole body intact, or appendages missing; D=abdomen; H=head; T=thorax; L=legs; A=antennae; R=body buried; or M=whole body missing.

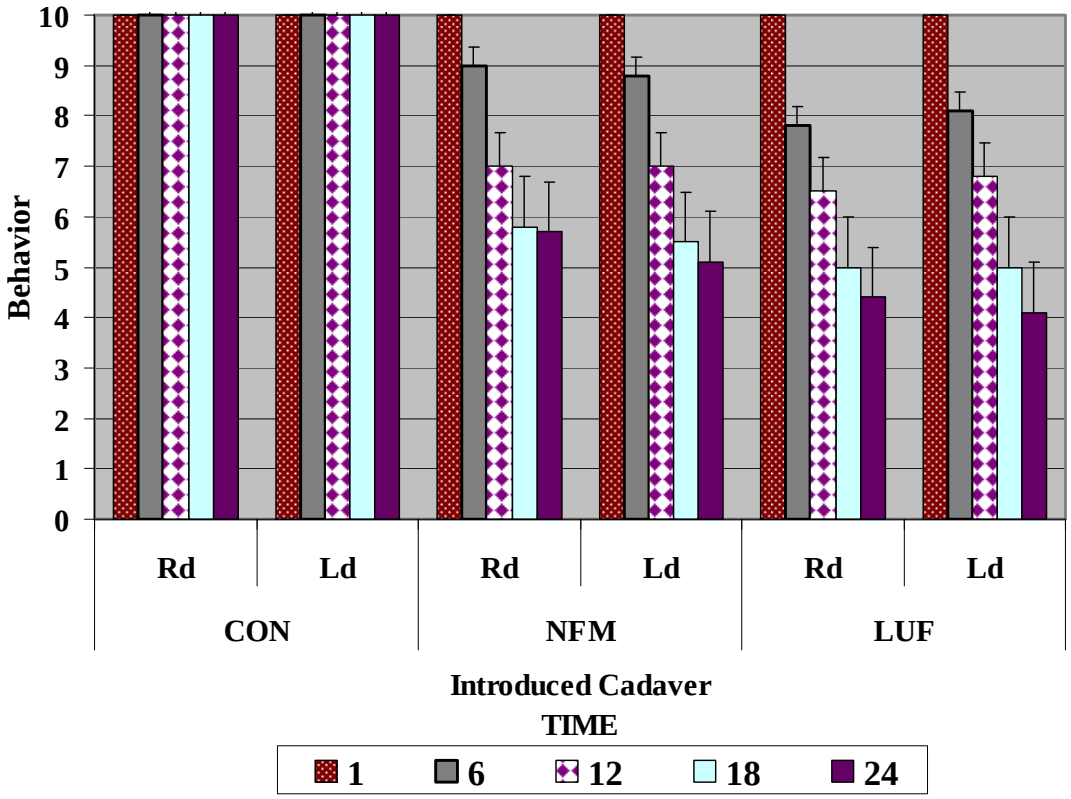

Figure 4.7. Behavioral range (0-10) based on removing Petri dish lid and observing *R. flavipes* worker response.

CHAPTER 5

THE IMPACT OF CSI ACTIVE INGREDIENTS ON PROTIST COMMUNITY OF THE SUBTERRANEAN TERMITE *RETICULITERMES FLAVIPES* (ISOPTERA: RHINOTERMITIDAE)[1]

[1] Lewis, J.L. and B.T. Forschler. To be submitted to *Environmental Entomology*.

5.1 Abstract

The eastern subterranean termite, *Reticulitermes flavipes* (Kollar) were continuously exposed to different chitin synthesis inhibiting (CSI) active ingredients and the protist community from the hindgut quantified biweekly for 21 d. CSIs included five commercially available formulations including diflubenzuron, hexaflumuron, lufenuron, noviflumuron, and one experimental CSI. Our study showed termites exposed to CSI had a significant decrease of at least 30% in estimated total protist population after only 3 days, regardless of CSI exposure. Protist species impacted were *Dinenympha fimbriata*, *D. gracilis*, *Microjoenia fallax*, *Pyrsonympha vertens*, and *Trichonympha agilis*. We also provide evidence lufenuron is highly toxic and discuss some of the implications this might have on termite.

5.2 Introduction

Chitin synthesis inhibitors (CSIs) are a current and highly commercialized method of termite population management (Su 2003). It is a baiting system comprised of benzoylphenyl ureas presented in an edible cellulose matrix that interfere with an insect's physical development and cuticle sclerotization during molting (Cohen 1987). This system relies on foragers picking up a toxic dose that is slow acting (within weeks), nonrepellent and transferable, thus, providing the potential of wide distribution within a termite population before the onset of acute toxicity or avoidance behavior. Toxicant-laden baits can be introduced through using the termite's own behavioral acts, like consumption, social grooming, and food sharing (Randall and Doody 1934, Beard 1974). The sharing of food can be either from the mouth or by proctodeal trophallaxis, consisting of hindgut liquid containing symbiotic microbes (Cleveland 1924, 1925a).

The majority of cellulose digestion and absorption in termites, unlike most insects, occurs in the hindgut, or paunch (Inoue et al. 1997). Many of the symbionts in the hindgut are cellulolytic and assist the insect host by breaking down partially digested cellulose and glucose important for termite nutrition. However, they differ in there sensitivity to oxygen (Cleveland 1924, 1925a-c, 1928, Trager 1934, Hungate 1938, 1939). The hindgut protist community is lost every time the insect molts, accounting for some of the tremendous variability between individuals. Nevertheless, the relative proportions are consistent and allow for comparison between populations (Kirby 1937, Honigberg 1970, Lewis and Forschler 2004b).

Toxicants can target either the termites or their microbial symbionts, which are essential for termite survival, and have included palatable baits containing antibiotics or wood toxins (Mannesmann 1972, Mauldin et al. 1981, Waller 1996, Cook and Gold 2000). Even though the symbiotic protists found in termites do not contain chitin, they live inside the termite's digestive tract lined with chitin and are shed along with the peritrophic matrix during molting. Because CSIs weaken the integrity of the peritrophic matrix (Lehane 1997, Morales-Ramos et al. 2006), it is during this process we speculated, the gut would increase in oxygenation and therefore, potentially impact the anaerobic protist community.

In this study, we investigated the impact of several CSI baits by quantifying the protist communities from *R. flavipes*. Termites were exposed to five commercially available CSIs including diflubenzuron, hexaflumuron, lufenuron, noviflumuron, and one experimental CSI for 21 d. We hypothesized that only those protist species that are sensitive to oxygen exposure will decrease from termite exposure to CSIs. We also predict that oxygen insensitive but cellulolytic protists will not change and they should be similar among the different CSIs studied.

5.3 Materials and Methods

Insects. Three populations of *R. flavipes* were sampled from field sites, separated by at least 100 m, in Whitehall Forest, Clarke County, Georgia and brought back to the laboratory intact within their food source (Table 5.1; Pop1a-b, Pop2, Pop3). Termites were identified to species using published keys to the soldier caste (Scheffrahn and Su 1994). Termites were collected using moistened corrugated cardboard and placed into a plastic containers (26.99 X 19.37 X 9.52 cm) with weathered pine wood slats (approx. 12.5 X 2.54 X 0.2 cm) in complete darkness inside an environmental chamber (27° C, ≥90% RH) until their use in bioassay (Forschler and Townsend 1996).

Approximately 460-475 by weight (fourth instar or older) worker termites were placed into a Petri dish (100X25 mm) with 20 grams of sand moistened with 3.2 ml of distilled water and a known weight (7.0 ± 1.0g) of commercially available α-cellulose tablet containing either no CSI (control), diflubenzuron 0.25% (Whitmire Micro-Gen, St. Louis, MO), hexaflumuron 0.50% (Dow AgroSciences, Indianapolis, IN), noviflumuron 0.50% (Dow AgroSciences, Indianapolis, IN), lufenuron -treated corrugated cardboard (1.0 ± 0.3g at 0.15%; Syngenta Corporation, Greensboro, NC), or an experimental CSI (Whitmire Micro-Gen, St. Louis, MO), for 21 d. Because of time constraints, Pop1 was set up at two separate times with Pop1a set up with the treatments hexaflumuron and noviflumuron and Pop1b with diflubenzuron, lufenuron and the experimental CSI (Table 5.1).

Measurements of Protist Populations. Termite hindguts were removed as previously described by Lewis and Forschler (2004a). The gut contents from five termites were pooled to form a sample and homogenized in 250 µl Trager U saline solution (Trager 1934) pH 6.8-7.2 sparged with a nitrogen gas mixture (N2 92.5%, H2 2.5%, and CO2 5%) at 1 liter per min for

five min. An anoxic saline solution allows more time for quantifying the anaerobic protists in the hindgut (Lewis and Forschler 2004a). Ten µl of the resulting solution was loaded onto a hemacytometer (Neubauer, Brightline, Horsham, PA) and protists identified and counted from 0.1 µl. This was replicated three times twice a week per treatment for 21 d (0, 3, 7, 10, 14, 17, and 21 days) and termite population.

Statistical Analysis. Data were compared using SAS-JMP (version 7.0) statistical software (SAS Institute, Inc., Cary, NC). For comparison of all termite populations, variables analyzed included total protist population and relative species abundance and evaluated with analysis of variance (ANOVA) and means separated by Tukey-Kramer honestly significant difference (HSD) test for multiple mean comparisons ($\alpha = 0.05$).

5.4 Results

All protist species previously described from *R. flavipes* (Yamin 1979, Lewis and Forschler 2004b) were found in the 3 termite populations sampled, except *Trichomitus trypanoides* and *Pyrsonympha major* were absent from population 1 (Table 5.2).

Treatments from population 1 were set up on different dates (Pop1a and Pop1b) with comparable total protist populations at time 0, although relative proportions of a few protist species did differ (Table 5.1). Relative protist proportions of *Microjoenia fallax* were 1 ± 2% in Pop1a, significantly lowere than 10 ± 2% found in Pop1b ($F=35.63$; $df=1, 4$; $p=0.0040$). Population 1a had a greater relative proportion of *P. vertens* (13 ± 4%) than Pop1b (6 ± 1%) ($F=9.30$; $df=1$; 4; $p=0.038$). However, our findings were comparable to those previously published (Lewis and Forschler 2003b) and any differences show the natural variation found in

the hindgut protist community; therefore, we grouped Pop1a and Pop1b together to form Pop1 group (Table 5.2).

The total protist population of Pop1 was approximately 50% smaller than both Pop2 and Pop3 (Table 5.1; $F=86.24$; $df=2, 9$; $p<0.001$). Within *R. flavipes*, the predominate protist species is *D. gracilis*, with a relative proportion of 54% or greater in the termite populations examined (Table 5.2). We found Pop2 (70 ± 2%) and Pop3 (71 ± 5%) had a greater proportion of *D. gracilis* than Pop1 (Table 5.2; $F=14.12$; $df=2, 9$; $p<0.0017$). We also found proportion of *T. agilis* in Pop1 were 8 ± 3% of the total protist population and greater than 3 ± 1% from Pop2 and Pop3 (Table 5.2; $F=9.52$; $df=2, 9$; $p=0.0060$).

Total Protist Population. Termites from all treatments had a significant decrease in total protist population over time, even after just 3 d in bioassay (Fig. 5.1; $F=63.41$; $df=125$; $p<0.0001$). The greatest impact to the termite protist community were following 3 d CSI exposure to lufenuron with only 46 ± 10% of the total protist population present, as compared to nontreated control (87 ± 24%), diflubenzuron (61 ± 10%), hexaflumuron (64 ± 20%) and the experimental CSI (60 ± 20%) (Fig. 5.1: $F=17.66$; $df=17, 39$; $p<0.0001$). Termites from the nontreated control decreased in total protist population to 72 ± 37% by 18 d, and were greater than 28 ± 13% from hexaflumuron, 26 ± 2% noviflumuron, and 6±9% lufenuron, but not different from (59 ± 29%) termites exposed to the experimental CSI (Fig.5.1; $F=39.40$; $df=17, 39$; $p<0.0001$). By 21 d, termites exposed to any of the CSIs had significantly smaller protist population than nontreated control (58 ± 22%), except for diflubenzuron with (57 ± 28%) (Fig. 5.1; $F=42.19$; $df=17, 39$; $p<0.0001$).

Relative Protist Species Proportions. Protist species proportions of *Dinenympha fimbriata*, *D. gracilis*, *Microjoenia fallax*, *P. vertens* and *Trichonympha agilis* (Figs. 5.2A-E)

changed over time in termites exposed to CSIs tested (Figs. 5.3A-E). Those protists species that did not significantly change over time are not presented here, and include *Holomastigotes elongatum*, *Monocercomonas* sp., *P. major*, *Spirotrichonympha flagellata, Spironympha kofoidi* and *T. trypanoides*.

Dinenympha fimbriata. Compared to field collected termites (11 ± 4%), *D. fimbriata* proportions decreased significantly by 7 d within untreated control (7 ± 2%; F=5.07; p=0.0017), diflubenzuron (6 ± 2%; F=5.21; p=0.0019), hexaflumuron (6 ± 2%; F=15.95; p=0.0001), and lufenuron (4 ± 4%; F=15.40; p<0.0001) treatments (Fig. 5.3A; df=9, 38). Proportions from termites exposed to the experimental CSI tended to increase during 1 wk (13 ± 8%) and 2 wk (15 ± 11%), but these findings were not statistically significant (Fig. 5.3A; df=9, 38; F=1.77; p=0.154). We did not see a significant impact on *D. fimbriata* in termites exposed to noviflumuron (Fig. 5.3A; df=9, 38; F=1.96; p=0.1199).

When comparing proportions of *D. fimbriata* between treatments, termites exposed to the experimental CSI had a greater protist proportion of *D. fimbriata* at 3 d (F=5.78; p=0.0003), 7 d (F=8.89; p<0.0001) and 14 d (F=6.00; p=0.0002), as compared to the other treatments (Fig. 5.3A; df=11, 45). However, by 21 d proportions of *D. fimbriata* were not different from termites exposed to the experimental CSI, than that of the nontreated control, diflubenzuron, and noviflumuron; yet were greater than hexaflumuron and lufenuron treatments (Fig. 5.3A; F=7.47; df=11, 45; p<0.0001).

Dinenympha gracilis. The most common protist species, *D. gracilis* (62 ± 8%), did not differ significantly within the untreated control (F=1.01; df=6; p=0.4130) and diflubenzuron (F=1.79; p=0.1512) treatments over time (Fig. 5.3B; df=9, 38). However, proportions of *D. gracilis* decreased in termites exposed to lufenuron by 7 d (F=14.36; p<0.0001), hexaflumuron

(F=17.27; p<0.0001) and noviflumuron by 14 d (F=9.63; p<0.0001), and the experimental CSI treatment by 21 d (F=2.83; p=0.0375), (Fig. 5.3B; df=9, 38).

Proportions of *D. gracilis* did not differ between treatments during the 1st week (3 d and 7 d), but by 14 d had decreased from termites continuously exposed to noviflumuron and lufenuron (Fig. 5.3B; F=2.86; df=11, 45; p=0.0253). By 21 d, approximately 50% less of the total protist population were *D. gracilis* from noviflumuron, hexaflumuron and lufenuron, compared to termites exposed to the nontreated control and diflubenzuron treatment (Fig. 5.3B; F=6.94; df=11, 45; p<0.0001).

Microjoenia fallax. Termites increased in protist proportions of *M. fallax* from 4 ± 4% by at least 50% at some time during the various treatments (Fig. 5.3C). By 7 d, termites from lufenuron exposure had increased to 22 ± 18% (Fig. 5.3C; F=14.34; df=9, 38; p<0.0001). Diflubenzuron (F=4.91; p=0.0027), hexaflumuron (F=4.53; p=0.0043), and noviflumuron (F=10.70; p<0.0001) treatments all had an increase in estimated proportion of *M. fallax* by 14 d (Fig. 5.3C; df=9, 38). The untreated control (F=4.10; p=0.0060) and experimental CSI (F=4.39; p=0.0052) both had an increase proportions of *M. fallax* at 21 d with 10 ± 7% and 16 ± 21%, respectively (Fig. 5.3C; df=9, 38).

We found lufenuron exposure had the greatest proportion of *M. fallax* by 7 d (F= 6.03; p=0.0002) compared to the other treatments (Fig. 5.3C; df=11, 45). However, by 14 d (F=4.86; p=0.0012) and 21 d (F=5.86; p=0.0003) noviflumuron exposure had a greater proportion of *M. fallax* than termites from both the control and lufenuron treatments (Fig. 5.3C; df=11.45).

Pyrsonympha vertens. Protist proportions of *P. vertens* were highly variable over time in all of the treatments and only differed in termites exposed to lufenuron at 21 d (2 ± 4%) (Fig. 5.3D; F=4.15; df=9, 38; p=0.0069).

Estimates of *P. vertens* only differed between treatments at 21 d, with a greater proportion from termites continuously exposed to noviflumuron (9 ± 9%) and hexaflumuron (10±7%) compared with lufenuron (2 ± 4%) (Fig. 5.3D; F=3.21; df=11, 45; p=0.0145).

Trichonympha agilis. We did not find proportions of *T. agilis* to change over time from termites in the untreated control (F=1.67; p=0.1710), diflubenzuron (F=0.57; p=0.6872), experimental CSI (F=0.83; p=0.5165) and noviflumuron (F=0.73; p=0.5741) treatments (Fig. 5.3E; df=9, 38). In contrast, estimated proportions from termites exposed to hexaflumuron increased from time 0 (6 ± 3%), to 12 ± 14% by 21 d (Fig. 5.3E; F=4.19; df=9, 38; P=0.0066). Estimates of *T. agilis* fluctuated from termites exposed to lufenuron, yet there was significantly greater proportion at 7 d (11 ± 8%), as compared with proportions undetectable at 21 d from termites sampled (Fig. 5.3E; F=6.38; df=9, 38; p=0.0005).

Proportions of *T. agilis* were highly variable but both hexaflumuron (12 ± 13%) and the untreated control (8 ± 5%) were significantly greater at 21 d, than that of (0%) lufenuron (Fig. 5.3E; F= 5.08; df=11, 45; p=0.0009).

5.5 Discussion

We observed all eleven protist species previously described from *R. flavipes* (Yamin 1979, Lewis and Forschler 2004b) from two of the termite populations, but *Pyrsonympha major* and *Trichomitus trypanoides* were not found at the volume sampled from Pop 1 (Table 5.2). Termites from Pop1 were maintained under laboratory conditions for two months prior to their use in bioassay, compared with only 4 and 5 weeks for Pop2 and Pop3, respectively (Table 5.1). The absence of these two termite protist species were noted by other researchers (Kirby 1937, Honigberg 1970, Lewis and Forschler 2004b) and could be explained by nutritional differences

(Smythe 1972), or their initial absence in the founding reproductive pair (Cleveland 1925c). However, Grosovsky and Margulis (1982) showed that these two protists species are not obligate symbionts in *R. flavipes* and therefore might be an unimportant indication of termite health. Another explanation for their absence might be that populations of these protist species were too low for detection and not necessarily absent.

Total protist population estimates from Pop1 (56,389) were similar to those previously reported from *R. flavipes* workers, averaging 58,369. However Pop2 (140,834) and Pop3 (122,500) were considerably larger (Lewis and Forschler 2004b) (Table 5.1). Protist species proportions were also similar to those previously described by Lewis and Forschler (2004b), except our Pop1 had greater proportion of *T. agilis* (8 ± 3% vs. 4 ± 2%) and Pop2 had less *S. kofoidi* (0.4 ± 0.4% vs. 2 ± 1%). When subterranean termites are removed from their habitat and brought into the laboratory, they are exposed to increased oxygen and a decrease in carbon dioxide (Lovelock et al. 1985, Curtis and Waller 1997) and over time, termites in captivity decrease in vigor (Arquette and Forschler 2006). This could contribute to the differences we saw in protist population estimates and their relative proportions from termite populations sampled. It is also possible differences were due to how the termites where collected from their original food resources. Termites become stressed by vibration (Grosovsky and Margulis 1982, Schwinghammer and Houseman 2006), and in previous studies, Lewis and Forschler (2004b) manually extracted termites from logs the day of bioassays. However, in our study we allowed termites to come out of logs at their own pace and a decrease in handling could explain why Pops2 and 3 had larger protist populations than have been previously reported.

Termites had a significant decrease in their protist population estimates in all treatments over time (Fig. 5.1). Our findings were in contrast with those reported by Perrott (2003), who

did not find a difference in total protist population estimates between untreated and hexaflumuron exposed termites. We believe these differences could be due to their protist counting techniques. Perrott (2003) used a simple saline solution (0.6M NaCl) which was later shown to make erroneous protist population estimates (Lewis and Forschler 2004a). Our protist counts were conducted using a saline solution sparged of any oxygen prior to use, to extend the life of these anaerobic symbionts following their removal from the termite hindgut.

Termites exposed to any of the five CSIs tested had at least a 30% decrease in their protist population estimates after only 3 d (Fig. 5.1). Because *D. gracilis* accounts for ~50% or more of the total protist community in *R. flavipes*, a significant decrease in this protist species would not be unexpected (Fig. 5.3B). Our results highlighted the total protist population decreased significantly in termites exposed to lufenuron treatment, and this CSI might be too toxic for effective management of termite population (Fig. 5.3A-E).

It could be inferred from our findings oxygen sensitive protists were more impacted by CSIs, than non-sensitive protist species. As has been documented previously, specific protist species play a distinct role in cellulose degradation and cellulolytic protist species from the hindgut of *R. flavipes* are *T. agilis* and *D. fimbriata*, based on the presence of wood fragments within food vacuoles, and the elimination of these protist species during starvation experiments (Cleveland 1925a, Grosovsky and Margulis 1982). However, only *D. fimbriata* are sensitive to oxygenation experiments (Cleveland 1925a, Grosovsky and Margulis 1982). A greater reduction in populations of the oxygen-sensitive species, *D. fimbriata* in termites treated with hexaflumuron and lufenuron (Fig. 5.3A and 5.3E) may suggest that those CSIs increase oxygenation in the hindgut.

In order to understand the complex interaction of these obligate symbionts in the termite host (Cleveland 1924, 1925a-c, 1928, Trager 1934, Hungate 1938, 1939), further research is needed. Current research in this field could benefit from new approaches by physiological characterization of the peritrophic matrix and how chitin content specifically impacts oxygen levels in the digestive tract. This information could provide valuable information in development of new termite baiting systems and their use in managing termite populations in and around human dwellings.

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Table 5.1. Termite collection, bioassay start date, and total protist population from *R. flavipes* worker.

		Date		Total Protists	
Pop[a]	Code	Collected	Start	Counted [b]	Population [c]
1a	Kooser	03.21.07	05.26.07	67.67±6.3a	56,389±5,293
1b	Kooser	03.21.07	06.14.07	67.67±6.1a	56,389±5,092
2	Log 10	07.24.07	08.21.07	169±21.7b	140,834±18,085
3	Log 21	09.28.07	11.09.07	147±10.4b	122,500±8,700

a Pop=termite population

b Mean (± SD) of protist cells counted within columns with the same lower case letter do not differ significantly ($p < 0.05$; $df = 3$; Tukey-Kramer HSD).

c Mean (± SD) total protist population from protist cell counts. Formula (#cells counted* volume guts homogenized in)/ (volume cells counted from*#termites).

Table 5.2. Protist species proportions from *R. flavipes* Pop1a, Pop1b, Pop2, and Pop3 at start of bioassays.

Species	Abbrev[a]	Protist Species Proportions[b]		
		Pop1	Pop2	Pop3
Dinenympha fimbriata	DF	14±4a	8±2a	9±3a
D. gracilis	DG	54±6a	70±2b	71±5b
Holomastigotes elongatum	HE	1±1a	1±1a	0.3±0.6a
Microjoenia fallax	MF	6±5a	3±0a	1±1a
Monocercomonas sp.	MO	0.2±0.4a	0a	0a
Pyrsonympha major	PM	0a	4±1b	4±1b
P. vertens	PV	10±4a	8±1a	6±2a
Spironympha kofoidi	DK	1±1a	1±1a	1±1a
Spirotrichonympha flagellata	SF	7±4a	4±1a	5±1a
Trichonympha agilis	TA	8±3a	3±1b	3±1b
Trichomitus trypanoides	TT	0a	<0.1a	<0.1a

a Abbrev= abbreviations of protist species

b Means (± SD) within a column followed by the same letter are not significantly different ($p<0.05$; df=3, 8; ANOVA).

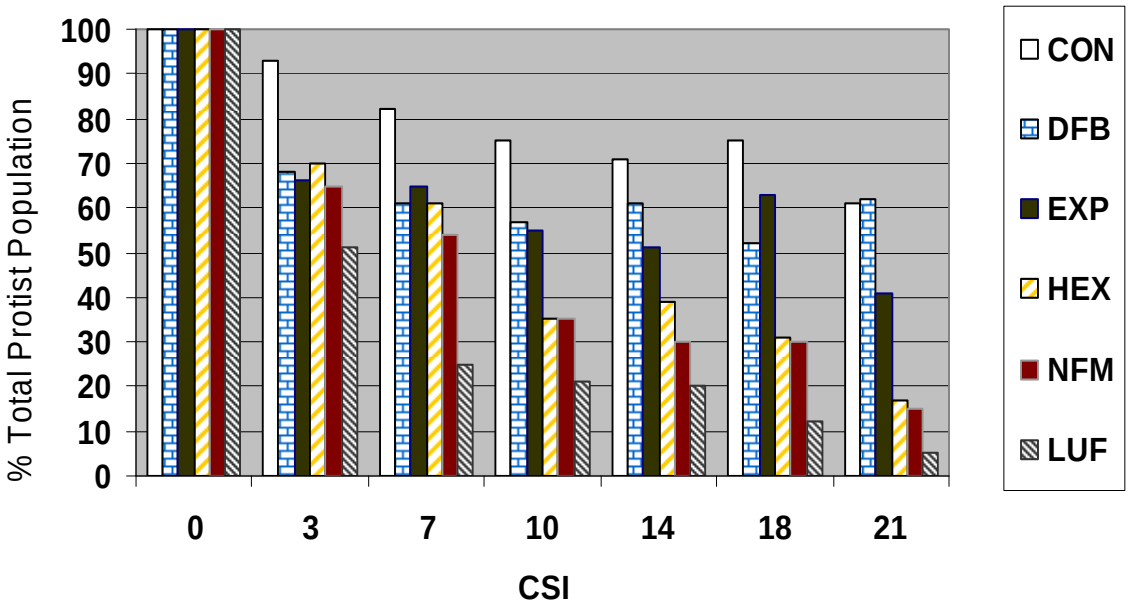

Figure 5.1. Average protist population from *R. flavipes* by treatment over time. Treatments include continuous exposure to CON=control; or CSIs DFB=diflubenzuron; EXP=experimental CSI; HEX=hexaflumuron; NFM=noviflumuron; or LUF=lufenuron.

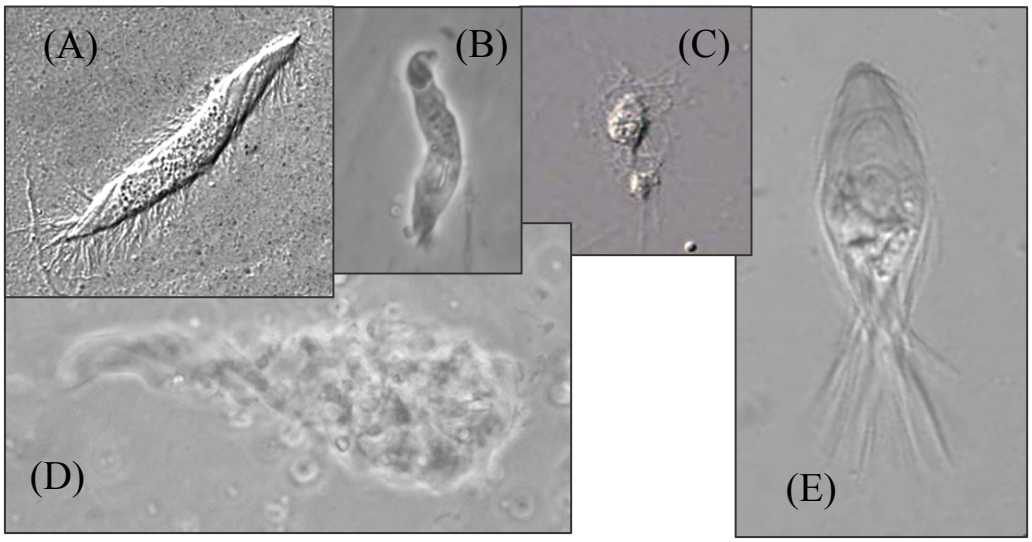

Figure 5.2. A-E. Protist species from *R. flavipes* that changed during exposure to chitin synthesis inhibitors. A) *Dinenympha fimbriata*. B) *Dinenympha gracilis.* C) *Microjoenia fallax*. D) *Pyrsonympha vertens*. E) *Trichonympha agilis*.

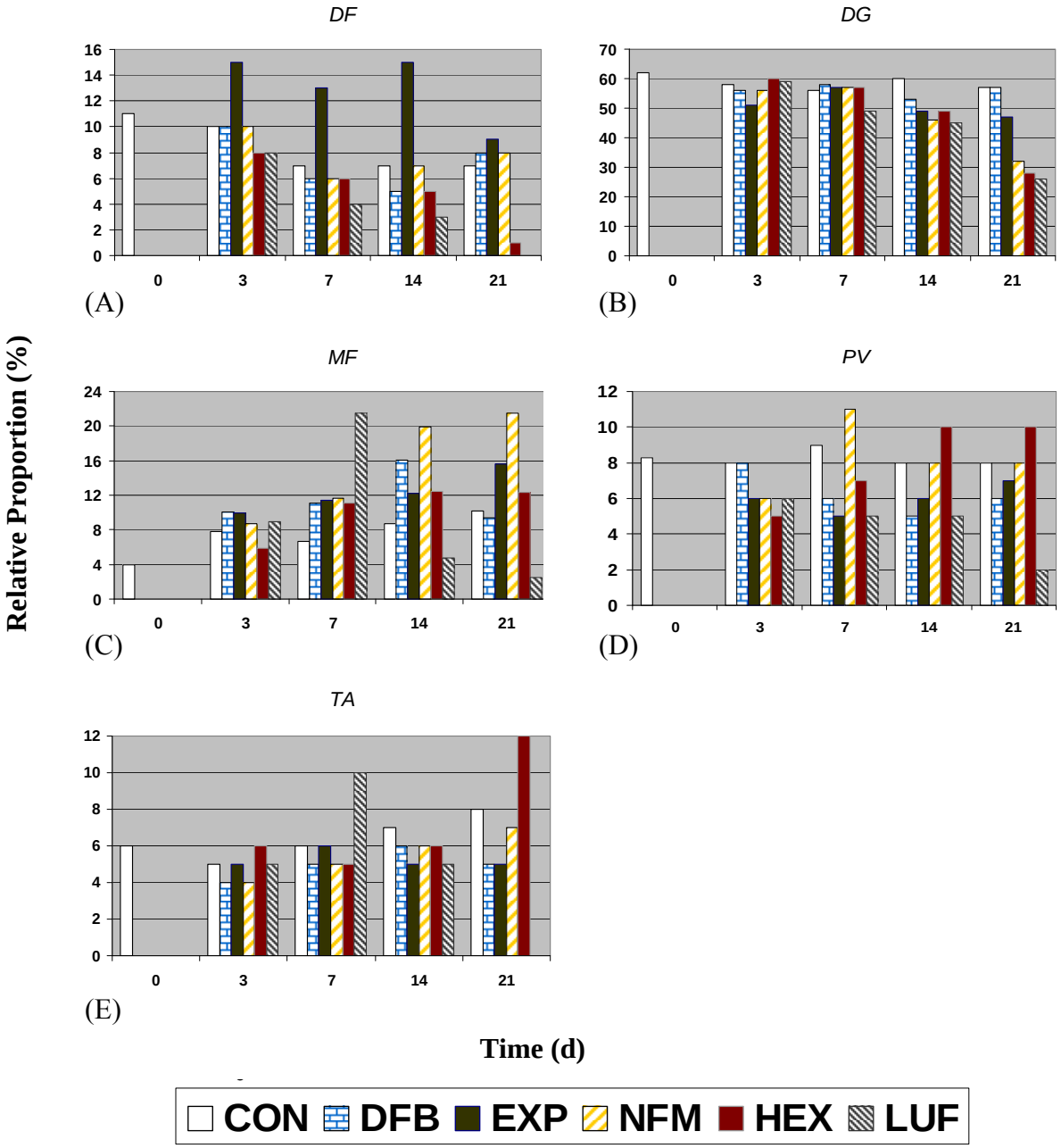

Figure 5.3. A-E. The effects of chitin synthesis inhibitors (CSIs) on protist species proportions from *R. flavipes*. A) DF=*Dinenympha fimbriata*. B) DG=*Dinenympha gracilis.* C) MF=*Microjoenia fallax*. D) PV=*Pyrsonympha vertens*. E) TA=*Trichonympha agilis*. Treatments include CON=control; or CSIs DFB=diflubenzuron; EXP=experimental CSI; NFM=noviflumuron; HEX=hexaflumuron; or LUF=lufenuron.

CHAPTER 6

SUMMARY

This research illuminated aspects of the mode of transfer, impact, and a comparative assessment of several commercialized CSI bait formulations. Efficacy of termite baiting systems are predicated on movement of the toxicant from the bait site to other locations within the network of galleries and feeding sites occupied by a subterranean termite population.. However, the method of CSI food-borne toxicants transfer is still not known.

Bioassays were designed to investigate toxicant transfer of CSIs by proctodeal trophallaxis at several donor to recipient ratios. My data showed CSI transferred at very low donor to recipient ratios resulting in high termite mortality. Termites were observed cannibalized and or buried even when cadavers were removed on a daily basis. This lead me to question the impact cannibalism has on toxicant transfer.

My research provided evidence subterranean termites will cannibalize their dead nestmates but also can detect "sick" individuals. Workers that had been exposed to CSI were subsequently buried. However, as evident by mortality, a toxic dose was transferred. Possible explanations for this avoidance might be explained by behavior. Termites that consume toxicant-laced food must be elicited for a food donation. Distribution of toxin could be compromised by workers that display signs of intoxication that are avoided (not solicited for a donation) and those that are unable to move from the CSI feeding site. Proctodeal trophallaxis also provides a method for termites to remain inoculated with symbiotic flagellates important in the insect host's health. There could be other impacts of CSIs other than just molting inhibition.

I quantified the symbiotic protist community from termites exposed to CSIs and found a significant decrease in the total protist population . These results raise questions about the role CSIs have on symbiotic microbes, and I predict because CSIs inhibit chitin synthesis, that this weakening of the peritrophic matrix could cause increase oxygenation of the gut. Possible implications could include oxygen sensitive protist species would be most affected. Further research could help elucidate these questions.