The biological activity of rare carbohydrates and cyclitols in Coptotermes formosanus

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THE BIOLOGICAL ACTIVITY OF RARE CARBOHYDRATES AND CYCLITOLS IN COPTOTERMES FORMOSANUS

A Dissertation

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

The Department of Biological Sciences

by

Lucas James Veillon
B.S., Louisiana State University, 2003
December 2011
ACKNOWLEDGEMENTS

I would like to thank my advisor and mentor Dr. Roger Laine, for introducing me to glycobiology and fostering my development as a researcher. His guidance has been indispensible and has opened many doors for my future. I would also like to thank Dr. Gregg Henderson for introducing me to entomology and supporting much of my research. Many thanks are also extended to my fellow Laine lab members Horia Negulescu and Dr. Betty Zhu, both were essential to my becoming oriented when I first joined the lab. I would also like to thank Dr. Brian Marx for his assistance with mathematical modeling and statistics. Finally I would like to extend my gratitude to committee members Drs. Grover Waldrop, Anne Grove and Jerome LaPeyre.
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ABSTRACT

In the interest of developing environmentally friendly interventions to infestations by Formosan subterranean termites, *Coptotermes formosanus* Shiraki, a number of carbohydrates were tested for effects on termites and symbionts. Among these, the D-galactose analog, 2-deoxy-D-galactose, *myo*-inositol and its fully phosphorylated derivative, phytate, showed promise as potential control chemicals. Feeding bioassays with 20 termite workers, where compounds were applied (concentrations ranged from 160.2-1281.7 µg/mm³) to 5 cm filter paper in water, indicated all three compounds significantly impact termite mortality in a dose dependent fashion over a 2 wk period. Interestingly, when *myo*-inositol was administered to termites in agar (40 mg/mL), in the absence of a paper food source, its toxic effect was abolished, while 2-deoxy-D-galactose toxicity remained, suggesting *myo*-inositol’s mechanism of toxicity may involve disruption of cellulose digestion and 2-deoxy-D-galactose may poison the termite directly. *Myo*-inositol feeding bioassays were also conducted with red imported fire ants, *Solenopsis invicta* Buren, who appeared immune to the toxic effect. Radiotracer feeding studies involving *myo*-inositol-[2-³H] and worker termites indicated *myo*-inositol is not chemically changed following its ingestion. When the hindgut protozoa of termites feeding on *myo*-inositol and 2-deoxy-D-galactose were quantified decreased populations were observed. *Myo*-inositol, phytate and 2-deoxy-D-galactose all take approximately one week to induce mortality, indicating that they may have promise as a delayed action toxins, which, if added to baits, could allow time after bait discovery for an entire colony to be affected.
TERMITES AS PESTS

Termites are a large group of organisms of which there are greater than 2600 characterized species in 281 genera (Kambhampati and Eggleton 2000). They are organized into seven families with 14 recognized subfamilies. The largest family, by far, is Termitidae which contains approximately 85% of described genera. Only a small fraction of the thousands of termite species described are known to cause damage to structures. Specifically, there are 183 known destructive species of which approximately 45% cause damage deemed significant (Kambhampati and Eggleton 2000). One hundred and forty seven of the known destructive species fall into the category of subterranean termites, of which Coptotermes spp., the focus of this work, are the most widely distributed.

Subterranean termite species, as their name implies, spend the majority of their lives underground. When their foraging areas overlap with man-made wooden structures a situation is created where they may become pests. Subterranean termites, including arboreal and mound building species, typically infest structures from the ground up. Aerial infestations carried out by alates also occur, but are less common (Su and Scheffrahn 2000).
TERMITE CONTROL METHODS

Control methods employed against subterranean termites include baits targeting colony populations, wood treatments, physical and chemical barriers (Su and Scheffrahn 2000). Chemical barriers may be created before the construction of a buildings foundation or after the fact. In either case the process involves the treatment of soil with liquid insecticide with the aim of excluding termites from the structure. While this is the most commonly employed control method, our compounds of interest are not applicable so it will only be discussed briefly.

Soil treatments have been widely used by commercial termite controllers for roughly a century. From the 1930’s through the 1950’s the liquid insecticides used included ethylene dibromide (dibromoethane), sodium arsenite, trichlorobenzene, pentachlorophenol, creosote and most infamously dichlorodiphenyltrichloroethane (DDT) (Randall and Doody 1934, U.S. Department of USDA 1951, Su and Scheffrahn 2000). Due to increased concern over the environmental impacts of insecticidal soil treatments, interest in the implementation of physical barriers has risen (Su and Scheffrahn 2000).

One example of physical barrier construction involves the utilization of uniform sized particles that are too large for termites to move and too small for them to pass between (Ebeling and Pence 1957, M. Tamashiro 1987, Smith and Rust 1990, Su et al. 1991b, Su and Scheffrahn 1992, Lewis et al. 1996). Another example is provided by stainless steel mesh barriers (TERMIMESH) (Lenz and Runko 1994, Grace 1996) and polymer sheets infused with insecticides, an example of a combination of both physical and chemical barrier methodologies (Lenz et al. 1998). Chemical and pressure
treatments with compounds such as creosote, pentachlorophenol or inorganic salts commonly required by building codes to prevent decay at the interface where soil meets wood, can be bypassed by subterranean termites and are thus largely ineffective. Local or “spot” treatments are employed to control active infestations but such treatments usually don’t reach the majority of the colony population and are also ineffective (Su and Scheffrahn 2000).

Population management approaches differ from the aforementioned methodologies in that they aim to reduce or eliminate nearby subterranean termite populations. As the research described herein relates directly to population management, the remainder of the review will focus on this area of termite control.

**INSECT GROWTH REGULATORS**

Juvenoids and chitin synthesis inhibitors constitute two classes of insect growth regulators that have been screened for activity against subterranean termites. Both cause gradual and cumulative mortality making them ideal for bait matrices (Su and Scheffrahn 2000).

**Chitin Synthesis Inhibitors.** Benzoylphenyl ureas inhibit chitin synthesis in arthropods, including insects and fungi (Hajjar and Casida 1978). In insects this inhibition leads to faulty endocuticular deposition and abortive molting (Ishaaya and Casida 1974, Post et al. 1974). Diflubenzuron [1-4(chlorophenyl)-3-(2,6-diflubenzoyl)urea], the first benzoylphenyl urea commercialized, alters the integrity of endocuticles (Grosscurt 1978, Grosscurt and Anderson 1980) by reducing the amount of chitin present. While studies have indicated that this reduction is a result of the inhibition of biochemical steps necessary for chitin production (Post et al. 1974, Van Eck 1979,

The first example of diflubenzuron inhibiting ecdysis in termites was provided by Doppelreiter and Korioth's (1981) experiments with *Heterotermes indicola* Wasmann and *Reticulitermes flavipes*. Further testing of diflubenzuron on *Microcerotermes* spp. field colonies yielded indefinite results (Faragalla et al. 1985) and laboratory testing of both diflubenzuron (Su and Scheffran 1993) and lufenuron (Su and Scheffrahn 1996), 1-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]-3-(2,6-difluorobenzoyl) urea, prevented ecdysis of *R. flavipes* but not *C. formosanus*. At the present only one benzoylphenyl urea, hexaflumuron, is known to effectively inhibit ecdysis in a wide range of subterranean termites species known to cause significant damage to wooden structures including *Reticulitermes, Heterotermes* and *Coptotermes* spp. (Su and Scheffran 1993, Su and Scheffrahn 1996, 2000).

The commercially available monitoring/baiting system known as Sentricon® uses hexaflumuron in its bait matrix. The system is organized such that when termites are

**Juvenoids.** Juvenile hormone analogs and juvenile hormone mimics constitute a class of insect growth regulators known as juvenoids. Interest in these compounds stems from the discovery that juvenile hormone regulates solider formation in termites (Luscher 1958, Luscher and Springhetti 1960, Lebrun 1967, Luscher 1969) and it’s known that soldiers are dependent on workers for feeding via trophallaxis. This knowledge sparked the idea that the collapse of a colony could be initiated by chemically inducing an excess of soldiers (Hrdy and Krecek 1972, Hrdy 1973, Haverty 1977, Su and Scheffrahn 2000).

Testing of fenoxycarb against field colonies of *Reticulitermes* spp. showed an increase in both pre soldiers and soldiers and a decline in foraging activity (Jones 1989). However, juvenoids only appear to be effective against species that naturally maintain a low proportion of soldiers (Su and Scheffrahn 1990). For example when methoprene and hydroprene were tested against a termite species that maintains
relatively high solider proportions, *Prorhinotermes simples* Hagen (Haverty 1977), increased solider proportions and a decline in colony population was not observed (Hrdy et al. 1979).

**METABOLIC INHIBITORS**

Another class of compounds that have been used in baits for the purpose of subterranean termite population management are metabolic inhibitors. Sulfluramid (Su and Scheffrahn 1988b, 1991, Henderson and Forshler 1996), diiodomethyl-p-tolyl sulfone (A-9248) (Su and Scheffrahn 1988a), dechlorane (mirex) (Esenther and Gray 1968, Esenther and Beal 1974, Ostaff and Gray 1975, Esenther and Beal 1978, Paton and Miller 1980), hydramethylnon (Su et al. 1982, Pawson and Gold 1996) and borates are all compounds in this class that have been used in termite baits. In field studies foraging activities and or colony populations were reduced when A-9248 (Su et al. 1991a), hydramethylnon (Su et al. 1991a, Pawson and Gold 1996) and sulfluramid (Su et al. 1995a) were examined. However, elimination of termite colonies in these studies was not observed.

Both the fact that boron is common in living organisms and that the tetrahydroxyborate ion \([\text{B(OH}_4^-]\)] can complex with any molecule containing two hydroxyl groups adjacent to one another has made elucidation of its mechanism of toxicity trying (Kim et al. 2004, Gentz and Grace 2006). Borates are often referred to as stomach poisons but research has demonstrated that termite mortality, at both high and low concentrations, occurs faster than the elimination of gut symbionts and ensuing starvation can account for alone (Khoo and Sherman 1979, Ahmed et al. 2004, Kartal and Ayrilmis 2005, Gentz and Grace 2006). In vitro studies have shown that borates at
high concentrations rapidly esterify to biomolecules of import, that to date have not been clearly identified. Data suggests that the borates are acting as metabolic poisons, causing toxicity through biostatic rather than biocidal mechanisms (Lloyd et al. 1990). The specific interaction of borates with numerous molecules of biological importance has been reported. These include nicotinamide adenine dinucleotide (NAD+), riboflavin, coenzyme A and vitamins B6 and B-12 (Lloyd et al. 1990, Williams et al. 1990, Woods 1994). An additional mechanism of toxicity possible for borates is presented by their action as ionic inhibitors, where they have been proposed to impact the stability of membranes and the polyols embedded within them (Lloyd et al. 1990).

Sulfluramid, N-ethylperfluorooctane-1-sulfonamide, is metabolized to perfluoroocatane sulfonamide (NDES), a N-deethylated analog (Hollingworth 2001). It is believed that sulfluramid is a propesticide that is readily converted to NDES which in turn acts as a mitochondrial uncoupler. Investigations with renal mitochondria indicate NDES causes a 50% increase in state IV respiration at 5 µM by acting as an ionophore consistent with it being considered a moderately active mitochondrial uncoupler (Schnellmann and Manning 1990, Hollingworth 2001). Such uncoupling activity is made possible by the fact that the sulfonamide group in NDES is weakly acidic, has a pKa of approximately seven in water and a high log P value (Hollingworth 2001). Additionally, because it has been demonstrated that fatty acids can act as protonophoric uncouplers with their anionic form being transported back across the mitochondrial inner membrane by a specific transporter system (Wallace and Starkov 2000) it has been proposed that NDES may demonstrate increased uncoupling potency through its binding to this transporter, thus acting as a fatty acid analog (Hollingworth 2001). Perfluoroocatane
sulfonic acid and its derivatives are also powerful surfactants (Shinoda et al. 1972, Kissa 2001), so their ability to disrupt the integrity of membranes should also be considered when evaluating their effects on mitochondria.

The precise mechanism of action for the banned compound mirex and its metabolites is unresolved. However, it has been suggested that it is a metabolic inhibitor and neurotoxic agent with a mechanism similar to DDT and other chlorinated hydrocarbon insecticides that open neuronal sodium channels (Minchew et al. 1980, Eisler 1985). Such a mechanism is characterized by the inhibition of ion channel inactivation and the shift of activation to hyperpolarized membrane potentials that result in repetitive discharges and synaptic malfunction ultimately causing hyperexcitability (Zlotkin 2001).

Hydramethylnon belongs to the amidinohydrazone chemical family. It disrupts cellular respiration in the mitochondria by inhibiting the electron transport chain at the cytochrome b-c (Hollingshaus 1987, NPIC 2002) complex starving cells of adenosine triphosphate. Hydramethylnon’s inhibition of the electron transport chain increases in a dose dependent fashion over time until cellular respiration is suppressed to the point of death (Hollingshaus 1987). Generally, after ingestion, insects appear lethargic after one day and die within three to seven days (Bacey 2000). Like several previously mentioned metabolic inhibitors its delayed mechanism of toxicity is favorable for pest controllers because it provides social insects, such as termites, enough time to spread the toxicant to other colony members.

A-9248 is also a slow acting compound that is non-repellent and a biocide. It’s known to be toxic to bacteria, algae and fungi as well as termites. While literature is
scarce on its mechanism of action in insects, the source of its toxicity in animals appears to be the iodine liberated during its metabolism (Saghir et al. 2011).

While some success has been achieved in the identification of termiticides, there is much work left to do. As human populations continue to grow they with indubitably drive an increase in the transport and utilization of lumber to meet construction needs, which will in turn facilitate the spread of termite species. Therefore, there is an ongoing need to discover slow-acting termiticides for inclusion in termite baits that will reduce or eliminate environmental impacts while improving efficacy. It is with this goal in mind that the experiments described within were conducted.

REFERENCES


Lenz, M., and S. Runko. 1994. Protection of buildings, other structures and materials in ground contact from attack by subterranean termites (Isoptera) with a physical barrier - a fine mesh of high grade stainless steel. Sociobiology 24: 1-16.


CHAPTER TWO

DELAYED ACTION TOXIC EFFECT OF 2-DEOXY-\textit{D}-GALACTOSE ON FORMOSAN SUBTERRANEAN TERMITES AND SYMBIONTS (ISOPTERA: RHINOTERMITIDAE)

INTRODUCTION

The Formosan subterranean termite, \textit{Coptotermes formosanus} Shiraki, a cellulophagous exotic species in the Southern USA, California and Hawaii, and endemic in Asia, infests and damages not only wooden structures, but over 50 species of living plants (Grace et al. 1996, Osbrink et al. 1999, Messenger et al. 2000, Lax and Osbrink 2003), and can also physically damage non-cellulosic materials such as buried electrical and telephone wires and insulation (Henderson and Dunaway 1999). Since the introduction of \textit{C. formosanus} to the continental US, populations have grown rapidly (Woodson et al. 2001, Lax and Osbrink 2003). In New Orleans, Louisiana alone the number of alates captured increased by a factor of 14 over a seven year period, illustrating the enormous potential for spreading throughout the Southern US (Henderson 1996).\(^1\)

It has been demonstrated that \textit{C. formosanus} possesses two separate cellulose-digesting systems, 1) endogenous endo-\(\beta\)-1,4-glucanase and \(\beta\)-glucosidase enzymes expressed in the salivary glands and midgut (Itakura et al. 1997, Nakashima et al. 2002, Zhu et al. 2005), and 2) from exogenous endo-\(\beta\)-1,4-glucanase and \(\beta\)-glucosidase enzymes secreted by symbiotic intestinal protozoa that aid in cellulose and lignin digestion (Yoshimura et al. 1993b, a, Itakura et al. 1997, Watanabe et al. 2002). The C.

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Tagatose, a rare sugar in nature, was shown to be toxic to fly maggots, and was commercialized as Flycracker® by Biospherics, Inc. (Levin and Zehner 1992, Spherix 2002). It has since been taken off the market and is being used as a human-use non-caloric sweetener. Levin’s observation led us to examine its effects on Formosan termites; however, tagatose had no effect in several dose-response experiments (Veillon et al. unpublished data). Therefore, we decided to examine a number of other rare sugars for termite toxicity (Appendices A1 – A10). Among these, 2-deoxy-\(D\)-galactose (Fig. 1) was the most active.

![Chemical structure of 2-deoxy-\(D\)-galactose](image.png)

**Fig. 1.** Chemical structure of 2-deoxy-\(D\)-galactose.
2-Deoxyhexoses are known inhibitors of cell growth and glycolysis in animal cells (Thirion et al. 1976, Smith and Keppler 1977, Zaret and Stevens 1990), yeast (Heredia et al. 1964, Platt 1984, Lagunas and Moreno 1992) and bacteria (Alper and Ames 1975, Thompson and Chassy 1982, Tanaka et al. 1994). In animal cells, 2-deoxy-\(\text{D}\)-galactose induces hepatotoxicity due to inhibition of uridine triphosphate dependent macromolecule biosynthesis by uridine phosphate depletion. Uridine trapping occurs when 2-deoxy-\(\text{D}\)-galactose is metabolized through the Leloir pathway to uridine 5-diphosphate 2-deoxy-\(\text{D}\)-glucose (UDPdGlc) resulting in the accumulation of both UDPdGlc and uridine 5-diphosphate 2-deoxy-\(\text{D}\)-galactose (Keppler et al. 1970). In higher plant cells, 2-deoxy-\(\text{D}\)-galactose has been reported to have a similar anti-metabolite activity resulting in the inhibition of synthesis of cell wall polysaccharides by decreasing the production of uridine diphosphate-sugars (Inouhe and Yamamoto 1991).

Slightly different mechanisms have been proposed for 2-deoxy-\(\text{D}\)-galactose toxicity in yeast and bacteria. In yeast cells, it has been suggested that glycolysis is inhibited by accumulation of 2-deoxygalactose-1-phosphate (dGal1P), which is thought to inhibit hexokinase and phosphofructo-kinase (Lagunas and Moreno 1992). However, in bacteria, fructose 1,6-bisphosphatase, important for gluco-neogenesis and amino sugar metabolism, is considered the site of dGal1P inhibition (Tanaka et al. 1994). With 2-deoxy-\(\text{D}\)-galactose’s established mechanisms of toxicity, we set out to examine if one or more, the termite itself, or both, could be affected by 2-deoxy-\(\text{D}\)-galactose and to establish its dose-response.
RESULTS

Preliminary Screening Assay.  *Delayed toxicity.* Mortality was significantly increased in workers fed filter paper treated with 320.4 µg/mm³ of 2-deoxy-D-galactose compared with the control (Fig. 2). On day seven, 25% of the workers feeding on treated filter had perished, after which the rate of mortality dramatically increased, to 100% on day 11 (Fig. 2). 2-deoxy-D-galactose-induced mortality became significantly different from controls on day 10 and beyond (Fig. 2).

Fig. 2. Means (±SE) of *C. formosanus* percent mortality following treatment of food source filter paper with 2-deoxy-D-galactose in no-choice assays with 20 workers (n=3). Colony A was used and the data were recorded daily for 14 d. Means followed by the same letter are not significantly different (*P* > 0.05). The *F* value for percent mortality is 15.47. *P* and df values are <0.0001 and 29, 60.
Dose-Response Feeding Assays. Bioassay 1: Dose-dependent toxicity. The 640.8 µg/mm$^3$ 2-deoxy-\(D\)-galactose filter paper treatment had the most significant impact on worker health resulting in 25% cumulative mortality after 5 d and 100% on day nine (Fig. 3). In this experiment, the 320.4 µg/mm$^3$ treatment caused a lesser but notable increase in worker mortality killing 25% of the workers by day nine and 53% after 14 d (Fig. 3). Increases in mortality were significantly different than the control following days five and 11, respectively, for the 640.8 and 320.4 µg/mm$^3$ doses (Fig. 3). Treatment of filter paper with 160.2 µg/mm$^3$ 2-deoxy-\(D\)-galactose did not significantly impact mortality (Fig. 3).

Bioassay 1: Decreased Paper Consumption. All three doses examined resulted in a statistically significant decrease in filter paper consumption. 2-deoxy-\(D\)-galactose treatment reduced filter paper consumption in a dose-dependent manner. During the 2 wk assay, each control worker consumed on average 0.13 mg of filter paper (Fig. 4). Individual workers feeding on filter paper treated with 2-deoxy-\(D\)-galactose consumed 90, 52 and 35% (respectively; 640.8, 320.4 and 160.2 µg/mm$^3$ treatments) less than the control (Fig. 4).

Bioassay 2: Dose dependent toxicity. Both the 1281.7 and 640.8 µg/mm$^3$ filter paper treatments significantly impacted worker mortality; however in this colony the 320.4 and 160.2 µg/mm$^3$ doses did not. Workers exposed to 1281.7 µg/mm$^3$ of 2-deoxy-\(D\)-galactose began to die faster than those exposed to 640.8 µg/mm$^3$, experiencing 25% mortality by day five compared to the 640.8 µg/mm$^3$ treatment groups 5% (Fig. 5). Despite temporal differences in the onset of toxicity, both the 1281.7 and
640.8 µg/mm³ treatments caused statistically significant increases in mortality following days five and seven, respectively (Fig. 5).

**Fig. 3.** Means (±SE) of *C. formosanus* percent mortality following treatment of food source filter paper with 2-deoxy-D-galactose in no-choice assays with 20 workers (n=3). Colony B was used and the data were recorded daily for 14 d. Means followed by the same letter are not significantly different (*P* > 0.05). The *F* value for percent mortality is 31.23. *P* and df values are <0.0001 and 59, 120.
Fig. 4. Means (±SE) of *C. formosanus* filter paper consumption per surviving termite over 14 days. Food source filter paper was treated with 2-deoxy-\(\text{D}\)-galactose prior to incubation. Colony B survival data were recorded daily for 2 weeks. Consumption data were recorded at the end of the 2 wk assay. Groups with statistically similar total consumption values bear the same letter (\(P > 0.05\)). The total consumption \(F\) and \(P\) values are 29.4 and 0.0001. The Df values are 3 and 8.
Fig. 5. Means (±SE) of *C. formosanus* percent mortality following treatment of food source filter paper with 2-deoxy-D-galactose in no-choice assays with 20 workers (n=3). Colony C was used and the data were recorded daily for 14 d. Means followed by the same letter are not significantly different (P > 0.05). The F value for percent mortality is 37.5. P and df values are <0.0001 and 74, 150.

**Intercolony Variation.** The effects of 640.8, 320.4 and 160.2 µg/mm³ doses, of 2-deoxy-D-galactose, were evaluated on multiple colonies. The results of an ANOVA followed by Tukey’s Studentized range test (SAS Institute 1989) indicated no intercolony variation in resistance, to 2-deoxy-D-galactose induced mortality, at the 160.2 µg/mm³ treatment level. However, at the 320.4 µg/mm³ treatment level the susceptibility of colony A (preliminary screening assay), to 2-deoxy-D-galactose induced mortality, was significantly different than that of colony B (dose-response bioassay 1) on days 11 through 13; and colony C (dose-response bioassay 2) on days 10 through 14.
Additionally, at the 640.8 µg/mm³ treatment level, colony B (dose-response bioassay 1) mortality was significantly different than that of colony C (dose-response bioassay 2) on days seven through 11.

**Complementary Log-Log Modeling Of Pooled Mortality Data.** The results of time-concentration-mortality CLL modeling of *C. formosanus* are shown in Table 1. The β parameter from the maximum likelihood estimation in the CLL model is the slope value (β). The relationship between time, concentration and mortality using the CLL model for 2-deoxy-D-galactose against *C. formosanus* is presented with the observed mortality for comparison in Figure 6. CLL modeling resulted in a trend of accumulated mortality over time increasing with concentration, indicating the onset of toxicity is concentration dependent. LC₅₀ and LC₉₀ estimates with 95% confidence intervals for 2-deoxy-D-galactose against *C. formosanus* are shown in Table 2. The highest 2-deoxy-D-galactose concentration evaluated was 1281.7 µg/mm³ (20mg per 42mm filter paper), so concentrations exceeding this value are a result of extrapolation. Figure 7 depicts the LT₅₀ estimates for 2-deoxy-D-galactose against *C. formosanus*. Estimated LT₅₀ values decreased with increasing concentration (Fig. 7). The lowest concentration examined, 160.2 µg/mm³, resulted in <50% mortality, thus preventing a LT₅₀ estimate.

When mortality data were pooled and subjected to ANOVA followed by Tukey's Studentized range test (SAS Institute 1989), the 1281.7 µg/mm³ treatment group was the only group determined to be significantly different than the control on days three through six (Fig. 8). On day seven and days nine through 14, both the 1281.7 and 640.8 µg/mm³ treatment groups were found to be statistically different than controls (Fig. 8).
Table 1. Parameters used in evaluating CLL modeling of time-concentration mortality for *C. formosanus* workers subjected to 2-deoxy-\(D\)-galactose.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>df</th>
<th>Scaled Deviance</th>
<th>Mean Deviance</th>
<th>Slope ((\beta))</th>
<th>SE of (\beta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-deoxy-(D)-galactose</td>
<td>408</td>
<td>800.45</td>
<td>1.96</td>
<td>2.28</td>
<td>.2049</td>
</tr>
</tbody>
</table>
Fig. 6. Relationship between observed and CLL model predicted percentage mortality, 2-deoxy-\(\textit{D}\)-galactose concentration, and time for \textit{C. formosanus} workers.
Table 2. Estimates of LC$_{50}$ and LC$_{90}$ in µg/mm$^3$ with 95% confidence intervals at different periods for *C. formosanus* workers.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time, d LC$_{50}$ (95% limits)</th>
<th>Time, d LC$_{90}$ (95% limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-deoxy-β-galactose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>167099.7$^a$ (166988.4-167211)</td>
<td>1 560858.9$^a$ (560741.5-560976.2)</td>
</tr>
<tr>
<td>2</td>
<td>23287.2$^a$ (23215.7-23358.7)</td>
<td>2 78162$^a$ (78087.9-78236.1)</td>
</tr>
<tr>
<td>3</td>
<td>7890.0$^a$ (7823.0-7957.1)</td>
<td>3 26482.5$^a$ (26413.6-26551.4)</td>
</tr>
<tr>
<td>4</td>
<td>4806.0$^a$ (4740.0-4872.0)</td>
<td>4 16130.9$^a$ (16063.4-16198.4)</td>
</tr>
<tr>
<td>5</td>
<td>3250.8$^a$ (3185.4-3316.1)</td>
<td>5 10911$^a$ (10844.3-10977.7)</td>
</tr>
<tr>
<td>6</td>
<td>1967.7$^a$ (1902.9-2032.6)</td>
<td>6 6604.6$^a$ (6538.7-6670.4)</td>
</tr>
<tr>
<td>7</td>
<td>1200.4 (1135.9-1264.8)</td>
<td>7 4028.9$^a$ (3963.7-4094.2)</td>
</tr>
<tr>
<td>8</td>
<td>815.2 (750.9-879.5)</td>
<td>8 2736.2$^a$ (2671.3-2801.1)</td>
</tr>
<tr>
<td>9</td>
<td>582.2 (517.9-646.4)</td>
<td>9 1954.0$^a$ (1889.3-2018.6)</td>
</tr>
<tr>
<td>10</td>
<td>463.9 (399.7-528.1)</td>
<td>10 1557.1$^a$ (1492.6-1621.7)</td>
</tr>
<tr>
<td>11</td>
<td>384.6 (320.4-448.9)</td>
<td>11 1291.0$^a$ (1226.6-1355.5)</td>
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<td>12</td>
<td>317.7 (253.4-381.9)</td>
<td>12 1066.3 (1001.9-1130.6)</td>
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<td>13</td>
<td>275.5 (211.2-339.7)</td>
<td>13 924.6 (860.3-988.9)</td>
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<td>14</td>
<td>257.0 (192.8-321.3)</td>
<td>14 862.7 (798.4-927.0)</td>
</tr>
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$^a$Estimate is extrapolated as the highest concentration used was 1281.7 µg/mm$^3$. 
Fig. 7. Concentration dependent LT50 estimates for 2-deoxy-D-galactose on *C. formosanus* workers.

Additionally, the group treated with 320.4 µg/mm³ of 2-deoxy-D-galactose was judged as significantly different than the control on days 12 through 14 (Fig. 8). The 160.2 µg/mm³ treatment group was determined to be statistically similar to controls throughout the duration of the experiment (Fig. 8).
Fig. 8. Means (±SE) of pooled *C. formosanus* percent mortality following treatment of food source filter paper with 2-deoxy-\(D\)-galactose in no-choice assays with 20 workers. Colonies A, B and C were used and the data were recorded daily for 14 d. Means followed by the same letter are not significantly different (\(P > 0.05\)). For controls, 160.2, 320.4, 640.8 and 1281.7 µg/mm\(^3\) treatment groups, values are 9, 6, 9, 6 and 3, respectively. The \(F\) value for percent mortality is 17.71. \(P\) and df values are <.0001 and 74, 420.

**Protozoa Counting.** Feeding on filter paper treated with the highest dose examined during dose-response feeding assays, 1281.7 µg/mm\(^3\) of 2-deoxy-\(D\)-galactose, resulted in decreased populations of all three protozoan species examined. *P. grassii* populations decreased the most followed by *H. hartmanni* and *S. leidyi* (Fig. 9). Total hindgut protozoan populations of 2-deoxy-\(D\)-galactose exposed workers were
significantly lower than controls on days five, six and seven (data collected on day five were significantly lower than controls on days three, six, and seven; data collected on days six and seven were significantly lower than controls on days two through eight) (Fig. 9). *P. grassii* populations were significantly decreased after day three (day three data were significantly lower than controls on days two, three and four; data collected from day four on were significantly different than controls on every day) (Fig. 9). *H. hartmanni* populations were significantly reduced on days three through 10 (data collected on days three, four and seven were significantly lower than controls on days two, six and eight; data collected on day eight were significantly different than controls on days two through four and days six through 10; data collected on days six and nine were significantly lower than controls on days two through 10; data collected on days five and 10 were significantly different than controls on every day) (Fig. 9). The observed decrease in *S. leidyi* population was found not to be statistically significant on any day. The experiment was planned for 14 d, however, because of 100% mortality in one 2-deoxy-\(D\)-galactose replicate, data could only be compared up to day 10.

**DISCUSSION**

2-deoxy-\(D\)-galactose toxicity to the Formosan subterranean termite was investigated. When applied to filter paper disc at 320.4 to 1281.7 \(\mu g/mm^3\), (depending on colony susceptibility) 2-deoxy-\(D\)-galactose significantly decreased feeding behavior and increased mortality (Figs. 2, 3, 4 and 5). Workers exposed to the sugar appeared lethargic and moved very little proximal to death.
Fig. 9. Means (±SE) of C. formosanus hindgut protozoan counts. The hindgut protozoa of workers from Colony D were enumerated daily for 10 d while termites were allowed to feed on filter paper treated with 1281.7 µg/mm³ of 2-deoxy-D-galactose. Means followed by the same letter are not significantly different (P > 0.05). The F values for total protozoa, Pseudotrichonympha, Holomastigotoides and Spirotrichonympha are 5.42 (P = 0.0002), 18.69 (P < 0.0001), 11.54 (P < 0.0001) and 2.78 (P = 0.0141), respectively. The df value is 19, 20 for all.

Because 2-deoxy-D-galactose exposure results in diminished feeding behavior it is conceivable that there is a correlation between starvation and mortality.

However, starved C. formosanus termites have been shown to live at least 12 d without significant mortality (Ibrahim et al. 2004), therefore it seems improbable that starvation is the cause. At this point we do not know if 2-deoxy-D-galactose is acting as a feeding deterrent or if the decreased paper consumption observed is a result of an overall reduction in termite health. Only choice bioassays, which we did not perform will
elucidate more conclusive information on feeding deterreny as a possible cause of reduced paper consumption. To date the use of carbohydrates as termite feeding deterrents has not been reported. Actually, quite the opposite, various carbohydrates have been suggested to act as termite phago-stimulants (Mishra 1992, Perrott et al. 2005, Zhou et al. 2008). Specifically, galactose has been reported to significantly increase *Reticulitermes* spp. consumption of paper baits (Swoboda 2004). Future research should evaluate mixtures of galactose and 2-deoxy-D-galactose in future experiments.

Hypotheses to explain 2-deoxy-D-galactose’s toxic effect on *C. formosanus* include the depletion of nucleotide pools, phosphate trapping or the build up of toxic secondary metabolites as discussed above. Additionally, 2-deoxy-D-galactose toxicity affecting any one of the numerous species of yeast, protozoa or bacteria living in the termite gut could disrupt its multi-organism dependent symbiotic environment, which could, in turn, induce termite mortality. The plausibility of such a “death by death of symbionts” hypothesis was initially supported by the finding that termites stripped of protozoa, by rearing on artificial agar substrates, experience significant mortality when provided a cellulosic food source (Tanaka et al. 2006). However, subsequent experiments conducted and described in chapter three do not support this mechanism of toxicity.

Since Baits for termites can have a form factor for example, a perforated plastic tube with rolled up cardboard containing 2-deoxy-D-galactose, and other insects are not cellulophagic, significant effects on other life forms are minimized. We believe this approach may provide another effective method for *C. formosanus* mitigation.
MATERIALS AND METHODS

**Insects.** Worker *C. formosanus* were collected from Brechtel Park, New Orleans, Louisiana as described by Smith et al. (2004). Bait crates (open-mesh plastic containers) were filled with southern yellow pine or spruce-pine-fir sapwood arranged into a lattice. Each crate was buried near a *C. formosanus* infested tree and collected after three to nine wk. Termite-infested crates were held in 250-liter cans with the original food source, and kept at room temperature (26-28 °C) and 70-80% relative humidity. Termites were collected from New Orleans on 14 May 2005 (colony A, preliminary screening experiment); 13 June 2006 (colony B, dose-response feeding experiment); 9 August 2006 (colony C, dose-response feeding experiment); and 14 September 2006 (colony D, protozoa counting experiment). Termites were extracted from the cans by removing wood sticks and tapping them into clean plastic containers where they were separated from unwanted material by collecting on moistened paper towels.

**Chemicals.** Neutral Red dye and 2-deoxy-\(D\)-galactose were obtained from Sigma-Aldrich (St. Louis, MO). The chemical structure of 2-deoxy-\(D\)-galactose is shown in Fig. 1.

**Preliminary Screening Assay.** 2-deoxy-\(D\)-galactose was screened for insecticidal activity (Fig. 1). Five milligrams of 2-deoxy-\(D\)-galactose dissolved in 25 µl of distilled water (dH₂O) was applied to a 42.5 mm filter paper, in a 60 x 15 mm plastic Petri dish, resulting in a concentration of 320.4 µg/mm³. Control filter paper received 25 µl of dH₂O. To prevent desiccation 100 µl of dH₂O was applied to the filter papers on the first day, after which 50 µl of dH₂O was applied every third day. Twenty worker
termites from colony A were incubated in the dark in a Petri dish at room temperature for 2 wk. Termite mortality was recorded daily for 14 d. An analysis of variance (ANOVA) (SAS ANOVA procedure) followed by Tukey’s Studentized range test was used to evaluate statistical differences among groups (SAS Institute 1989). All mortality data were judged at α = 0.05. All experiments were performed in triplicate.

**Dose-Response Feeding Assays.** 2-deoxy-\(D\)-galactose was screened for toxicity. Experimental methods were identical to those described for the preliminary screening assay with the addition that filter papers were weighed for consumption prior to sugar application and the effects of 160.2, 320.4, 640.8 and 1281.7 µg/mm\(^3\) of 2-deoxy-\(D\)-galactose were examined. When applying 2-deoxy-\(D\)-galactose to filter paper, at the beginning of each experiment, a solution consisting of 1 mg of sugar per 5 µl of dH\(_2\)O was always used. dH\(_2\)O was subsequently added to bring the total volume of liquid applied to the paper to 125 µl. After 14 d termites were removed from the dishes and the filter paper from each replicate was cleaned, washed of residual carbohydrate and dried at 100 °C for 24 h. After drying, filter papers were reweighed to determine consumption. Two assays were designed. In the first assay, the effects of 160.2, 320.4 and 640.8 µg/mm\(^3\) doses were examined on workers from colony B. In the second, using workers from colony C, the effect of an additional 1281.7 µg/mm\(^3\) dose was evaluated. Mortality and consumption data were subjected to ANOVA followed by Tukey’s Studentized range test (SAS Institute 1989). All data were judged at α = 0.05. All experiments were performed in triplicate.

**Complentary Log-Log Modeling.** A serial time-concentration-mortality design was implemented (Robertson and Preisler 1992). Conditional mortality probability was
estimated using the complementary log-log (CLL) model described by Robertson and Preisler (1992). SAS PROC GENMOD (SAS Institute 1989) was used to obtain maximum likelihood estimates of the conditional response parameters, which were in turn used to estimate cumulative mortality probabilities (Robertson and Preisler 1992). A small positive amount was added to all concentration levels to include controls (Robertson and Preisler 1992). LC50 and LC90 values with confidence limits were estimated using the formulae given by Robertson and Preisler (1992) and LT50 values were estimated by linear interpolation (Nowierski et al. 1996, Feng et al. 1998).

Additionally, pooled mortality data were subjected to ANOVA followed by Tukey’s Studentized range test (SAS Institute 1989). All data were judged at $\alpha = 0.05$.

**Protozoan Counting.** 2-deoxy-\textit{D}-galactose’s effect on \textit{C. formosanus} hindgut protozoan populations was examined. Twenty milligrams of 2-deoxy-\textit{D}-galactose dissolved in 100 μl of dH$_2$O was applied to filter paper, in a 60 x 15 mm plastic Petri dish, yielding a concentration of 1281.7 µg/mm$^3$. Control filter paper received 100 μl of dH$_2$O. Seventy five worker termites, from colony D, were placed in each dish.

*Pseudotrichonympha grassii* Koidzumi, *Holomastigotoides hartmanni* Koidzumi and *Spirotrichonympha leidyi* Koidzumi were counted daily for 2 wk as described by Mannesmann (1972) and modified by Maistrello et al. (2002). Hindguts were removed from the posterior ends of three workers and gently macerated in 40 μl of saline solution containing neutral red dye (0.5 ml of 1 % aqueous neutral red solution dissolved in 10 ml saline solution). The number of each protozoan species was determined using a hemocytometer (Bright-line Improved Neubauer, Hausser Scientific, Horsham, PA) under a light microscope. The population of each protozoan species per hindgut ($X_F$)
was calculated as: \( X_F = \frac{(G \times n)}{(V \times 3)} \), where \( G = \) volume (\( \mu l \)) of solution hindguts dissected in; \( n = \) mean of two counts within hemocytometer; \( V = \) volume (\( \mu l \)) of area counted. Mean (\( \pm SE \)) \( X_F \) values calculated from two replicates were used for graphical comparison of data. Protozoan population data were subjected to ANOVA followed by a Student-Newman-Keuls test (SAS Institute 1989), all data were judged at \( \alpha = 0.05 \). A square root transformation was applied for data analysis, however untransformed means are reported.

REFERENCES


CHAPTER THREE

EFFECTS OF MYO-INOSITOL AND PHYTATE ON SUBTERRANEAN TERMITES (ISOPTERA: RHINOTERMITIDAE) AND THEIR HIND-GUT PROTOZOA

INTRODUCTION

Of the 28 species in the genus Coptotermes the Formosan subterranean termite, Coptotermes formosanus Shiraki, an invasive cellulophagic species introduced into the Southern USA in the 1950s, is the most destructive and economically important worldwide (Hardy 1988, Henderson 2001, Woodson et al. 2001, Messenger 2002, Su 2003). C. formosanus infestations damage wooden structures, over 50 species of living plants (Grace et al. 1996, Osbrink et al. 1999, Messenger et al. 2000, Lax and Osbrink 2003), and also gnaw on and damage non-cellulosic materials such as insulation on buried electrical and telephone wires (Henderson and Dunaway 1999). The first general survey of a C. formosanus infested region in the continental US was conducted in Louisiana in 1966 (Spink 1967) and subsequent studies have indicated that US populations are growing rapidly (Woodson et al. 2001, Lax and Osbrink 2003). An example of this rapid growth is provided by an investigation conducted in New Orleans, Louisiana that found termite alates, which are potential founders of new colonies, increased by a factor of 14 over a seven year period (Henderson 1996).

C. formosanus digest their wood diets by utilizing endogenous endo-β-1,4-glucanase and β-glucosidase enzymes expressed in the salivary glands and midgut (Itakura et al. 1997, Nakashima et al. 2002, Zhu et al. 2005), and exogenous endo-β-1,4-glucanase and β-glucosidase enzymes secreted by symbiotic hindgut protozoa that

Our interest in investigating carbohydrates as termiticides was originally sparked by the reports that $D$-tagatose, a rare sugar, was toxic to fly maggots, and was commercialized as Flycracker® by Biospherics, Inc. (Levin and Zehner 1992, Spherix 2002). Our initial investigations involved screening a number of rare sugars (Appendix A) including $D$-tagatose and 2-deoxy-$D$-galactose. While $D$-tagatose was found to have no effect in toxicity screening experiments (Appendix A2) 2-deoxy-$D$-galactose was found to significantly increase Formosan subterranean termite mortality while decreasing hindgut protozoan populations (Veillon et al. 2010). We also observed that *myo*-inositol-2-monophosphate bis-cyclohexylamine salt impacted termite mortality (Veillon et al. unpublished data), which subsequently led us to examine the effects of, the far less expensive but chemically related compounds, *myo*-inositol (Fig. 10) and phytate (Fig. 11).
Of the nine isomeric forms of cyclohexanehexol, *myo*-inositol is the isomer most common in biology, although, at least five others are found (*chiro*-, *epi*-, *muco*-, *neo*- and *scyllo*). In the cell *myo*-inositol is synthesized *de novo* from glucose-6-phosphate in a two-step enzymatic process. In all organisms that make *myo*-inositol, the first step is a NAD\(^+\)-dependent reaction catalyzed by evolutionarily related *myo*-inositol-3-
phosphate synthases (MIPS), where glucose-6-phosphate is cyclized to \(D\-\text{myo}\)-inositol-3-phosphate (Donahue and Henry 1981, Chen et al. 2000, Loewus and Murthy 2000, Majumder et al. 2003b, a, Daiyasu et al. 2005, Stieglitz et al. 2005). The final step, catalyzed by inositol monophosphatase (InsPase), is characterized by dephosphorylation of \(D\-\text{myo}\)-inositol-3-phosphate to free \(\text{myo}\)-inositol (Stieglitz et al. 2007).

The synthesis & utilization of \(\text{myo}\)-inositol derivatives is observed in organisms spanning all three Kingdoms of life. Genes encoding MIPS and InsPase are common in the Euryarcheota and Crenarchaeota, both major clades of Archaea, where \(\text{myo}\)-inositol is incorporated, as a headgroup, into the characteristic \(sn\)-1-phosphoryl-2,3-diether-based membrane glycerolipids of many archeons (Majumder et al. 2003a, Koga and Morii 2007, Stieglitz et al. 2007). Additionally, the use of the \(\text{myo}\)-inositol derivative, di-\(L\-\text{myo}\)-inositol-1,1'-phosphate, as a stress-adaptive compatible solute has been observed in some archaeons known to inhabit very hot environments where its accumulation is associated with surviving transient exposure to temperatures more extreme than typically found in their environments (Scholz et al. 1992). Accumulation of this thermoprotective solute is accomplished by the transfer of \(D\-\text{myo}\)-inositol-3-phosphate from \(D\-\text{myo}\)-inositol-3-phosphate-cytidine 5'-diphosphate to the third carbon of a free \(\text{myo}\)-inositol molecule (Chen et al. 1998). Comparatively, few bacteria utilize \(\text{myo}\)-inositol derivatives. Of those that do, the majority are either obligate hyperthermophiles belonging to the \(\text{Aquifex}\) and \(\text{Thermotoga}\) clades or \(\text{Mycobacterium}\) species in the actinobacterial clade. Bacteria of the \(\text{Aquifex}\) and \(\text{Thermotoga}\) clades are particularly unique, in terms of evolution, in that nearly twenty five percent of their genes
have been imported from Archaea (Boucher et al. 2003) and their acquisition of MIPS enzymes is believed to have occurred by lateral gene transfer from co-existing archeons (Nesbo et al. 2001). Additionally, the utilization of myo-inositol containing compounds either by means of *de novo* synthesis or the use of H⁺-driven anti-porters has been sporadically reported in other bacterial species (Fahey 2001, Nesbo et al. 2001, Majumder et al. 2003a, Chatterjee et al. 2004, Wright and Turk 2004, You et al. 2004, Deretic et al. 2007, Michell 2007), including some gram-negative ice nucleators in which it is debatable if ice-nucleating proteins are glycosylphosphatidylinositol anchored on the bacterial cell surface (Kozloff et al. 1991, Jung et al. 1998, Jung and Pan 1998, Nosjean 1998), but such instances seem to be the exception and atypical.

All Eukaryotes, with few exceptions, use myo-inositol derivatives for many cellular processes. Of particular prominence is their use as protein anchors, membrane phospholipid components and polar hydrophilic solutes for adapting to challenges presented by the environment (Michell 2007). The rare eukaryotes that lack MIPS, and are incapable of synthesizing their own myo-inositol, are auxotrophs. In general, these organisms inhabit environments rich in inositolpolyphosphates & survive by secreting phytase enzymes and transporters to pump myo-inositol into their cells (Ingavale and Bachhawat 1999). An example of eukaryotic cells lacking cytosolic myo-inositol polyphosphates is provided by mammalian erythrocytes, which differ from the nucleated erythrocytes of reptiles, birds and fish that synthesize myo-inositol-1,3,4,5,6-pentaphospate (Val 2000, Villar et al. 2003). Some organisms, such as yeast, that utilize environmental myo-inositol are also capable of synthesis through transcriptional regulation of MIPS expression dependent on the environmental concentration of myo-
inositol (Carman and Kersting 2004, Nunez and Henry 2006). Although many metazoan cell types do not synthesize their own myo-inositol, in mammals, the testis, non-neural brain cells and some other tissue types are able to do so by utilizing cytosolic MIPS and InsPase (Parthasarathy and Eisenberg 1986, Tanaka et al. 1996, Antonsson 1997, Fisher et al. 2002, Chauvin and Griswold 2004, Michell 2007, 2008). Myo-inositol deficiency can be induced in rats and gerbils through means of dietary restriction, where lactating females are most susceptible (Holub 1982). The deficient state is characterized by an accumulation of low-density lipoproteins in the mucosa of the liver and or intestine (Holub 1982). The observation that this accumulation can be amplified by antibiotic treatment implies that some animals may acquire myo-inositol from their intestinal flora (Hayashi et al. 1974).

Of the many phosphorylated myo-inositol derivatives phytate, also known as phytic acid and myo-inositol-1,2,3,4,5,6-hexakisphosphate, is the most abundant. It’s accumulated in numerous plant organs and tissues including tubers, turions, roots and pollen, and is the principal form of phosphorus in seeds (Cosgrove 1980, Raboy 1997). Of the many putative functions of phytate in plant tissues perhaps the clearest is its use for storing and retrieving phosphorous, chelated calcium, other minerals and myo-inositol throughout plant development and germination. It is ubiquitous in eukaryotes and usually the most plentiful inositol phosphate derivative in cells (Sasakawa et al. 1995), where it and other phosphorylated myo-inositol derivatives are involved in numerous functions not directly related to the storage of nutrients.

Phytate synthesis in Dictyostelium and plants is well established, where phytate is derived from myo-inositol via the formation of the intermediate D-myoinositol 3-
phosphate, which is sequentially phosphorylated by positionally selective kinases (Irvine and Schell 2001). In mammalian cells the method of phytate synthesis is not entirely clear. However, it appears that its synthesis may be accomplished through the action of an inositol polyphosphate multikinase (mIPMK) (Saiardi et al. 2001) that sequentially phosphorylates \textit{myo}-inositol-1,4,5-phosphate to form \textit{myo}-inositol-1,3,4,5-phosphate which is further phosphorylated to produced \textit{myo}-inositol-1,3,4,5,6-phosphate. \textit{Myo}-inositol-1,3,4,5-phosphate can also be formed through the action of a very specific \textit{myo}-inositol-1,4,5-phosphate 3-kinase that is thought to have evolved from mIPMK and is enriched in neural tissue (Irvine and Schell 2001). Unequivocal evidence for the existence of an \textit{myo}-inositol-1,3,4,5,6-phosphate 2-kinase, the final step needed to complete the synthesis of phytate, has yet to be provided in mammals, but the existence of such an enzyme has been shown in yeast and maize (Ives et al. 2000, Sun et al. 2007).

Wide arrays of physiological functions and effects have been proposed for phytate. These include proposed anti-neoplastic effects in non-neural tissues (Jariwalla 1999), cellular antioxidant activity (Graf et al. 1987), the ability to prevent kidney stone formation (Grases et al. 1998), the regulation of stomatal pore closing in plants (Lemtiri-Chlieh et al. 2000), DNA repair activation (Hanakahi et al. 2000), coordination of nuclear mRNA transport (York et al. 1999, Saiardi et al. 2000, Feng et al. 2001), protein kinase C and protein phosphatase regulation, (Efanov et al. 1997, Larsson et al. 1997, Shears 2001, Fisher et al. 2002) in addition to roles in the central nervous system. Large numbers of high affinity phytate binding sites have been detected in membrane preparations from diverse brain regions (Sasakawa et al. 1995) and audioradiographic
studies suggest they are localized to the soma of neurons (Fisher et al. 2002). In addition to neuronal cell bodies phytate has been shown to bind strongly to myelin proteolipid protein, L-type calcium channels (Larsson et al. 1997, Fisher et al. 2002) and numerous other purified proteins, many of which are associated with membrane trafficking events. These include synaptotagmin (Fukuda et al. 1994), arrestins (Gaidarov et al. 1999) and the vesicle adaptor proteins AP-180 (Ye et al. 1995) and AP-2 (Voglmaier et al. 1992). All of which have contributed to speculation that phytate may play a role in the release of neurotransmitters (Llinas et al. 1994, Ohara-Imaizumi et al. 1997) as well as vesicle trafficking (Gaidarov et al. 1996) and receptor regulation (Sasakawa et al. 1995). It is important to note that because of the high negative charge phytate possesses it has a tendency to bind non-specifically and caution should be exercised when accepting putative functions, particularly if the criteria proposed by Shears (2001) to differentiate phytate specific from phytate non-specific roles have not been followed.

A considerable amount of effort has been devoted to the study of the dietary requirement for myo-inositol in insects. Of the phospholipids of insects phosphatidylcholines are among the most prominent and are the major phospholipid in all taxa studied except the order Diptera, where phosphatidylethanolamines are most abundant. Phosphatidylinositols have been identified in comparatively few insects and are always minor components of the phospholipid fraction (Fast 1964, 1970). A requirement for myo-inositol by some stored-products-consuming insects has been reported. However, because dietary restriction only retarded development towards pupation and didn’t significantly reduce adult numbers and most insects studied have
shown no need for myo-inositol these claims are indefinite (Dadd 1985). The first unquestionable insect dietary myo-inositol requirement was demonstrated in Periplaneta American, the cockroach, (Forgash 1962) which grew well on an myo-inositol deficient diet for a few instars but then lagged in growth with increased mortality resulting in few individuals reaching mature adulthood. Afterwards myo-inositol was found to be essential for all Orthoptera, the grasshopper order, examined (Dadd 1973). Additionally, myo-inositol has been found to be essential for several Lepidoptera, the butterfly and moth order, and Coleoptera, the beetle order, (Vanderzant 1959, Horie et al. 1966, Vanderzant 1968, Galford 1972), but certainly not all (Kasting and McGinnis 1967, Levinson et al. 1967, Rock 1969), as well as some aphids (Dadd et al. 1967, Ehrhardt 1968). It’s notable that several closely related species differ in their need, or lack thereof, for myo-inositol, an example of this is provided by the beetles Tribolium confusum and Tribolium castaneum (Pant and Gabrani 1963).

In Hymenoptera, myo-inositol is not required in synthetic diets for Itoplectis conquisitor (Yazgan 1972), a parasitoid, but is required by worker honey bees in artificial food for sustained larvae rearing (Nation and Robinson 1968). Contrastingly, none of the numerous larval Diptera studied seem to require dietary myo-inositol (Kleinjan and Dadd 1977), although it has been reported that its absence in adult fruit fly diets adversely impacts egg production (Tsiropoulos 1980).

Reports of dissimilar phosphatidylinositol metabolism in comparison with other phospholipids connected to salivary gland function (Berridge and Fain 1979, Fain and Berridge 1979) and muscle activity (Novakova et al. 1976, Helm et al. 1977, Strunecka et al. 1978) imply that phosphatidylinositols might be necessary for specialized
physiological functions in inositol-requiring and non-inositol-requiring insects alike (Dadd 1985). But, if a physiological need for myo-inositol is commonplace among all insects, it seems numerous taxonomically diverse species have lost their ability to derive it from glucose. It is notable that most insects found to require dietary myo-inositol have a habit of feeding on seeds or living plant tissue, which are both excellent sources of combined inositol (Dadd 1985). An example is provided by the boll weevil, who derives it from plant inositides and phytic acids (Vanderzant 1963).

With our initial observations that myo-inositol and phytate exhibited termite toxicity, and that relatively little is known about the biology of these compounds in Isoptera, we set out to establish their dose-response toxicity and characterize their impact on C. formosanus. Beyond assessing the direct impact on the termites themselves, an additional aim was to determine if one of more of the systems involved in the regulation of the C. formosanus intestinal hindgut community could be affected by myo-inositol and or phytate.

RESULTS

Dose-Response Feeding Assays. Myo-inositol (1281.7, 640.8 and 320.4 µg/mm³): Dose-dependent toxicity. Mortality was significantly increased in workers fed filter paper treated with both 1281.7 and 640.8 µg/mm³ of myo-inositol compared with the control (Fig. 12). Mortality became significant in both groups after d three, with the 640.8 µg/mm³ treatment inducing 100% mortality by d seven and the 1281.7 µg/mm³ treatment doing so by d five (Fig. 12). The 320.4 µg/mm³ treatment failed to induce significant mortality during the 14 d assay.
Fig. 12. Means (±SE) of *C. formosanus* percent mortality following treatment of food source filter paper with *myo*-inositol in no-choice assays with 20 workers (n=3). Collection group C was used and the data were recorded daily for 14 d. Means followed by the same letter are not significantly different (*P* > 0.05). The *F* value for percent mortality is 212.68. *P* and df values are <0.0001 and 74, 150.
Myo-inositol (1281.7, 640.8 and 320.4 µg/mm³): Decreased paper consumption. All three treatments examined resulted in a statistically significant decrease in filter paper consumption (Fig. 13). Myo-inositol treatment reduced filter paper consumption in a dose-dependent manner. During the 2 wk assay, each control worker consumed on average 0.162 mg of filter paper (Fig. 13). Individual workers feeding on filter paper treated with myo-inositol consumed an average of .027, .079 and .1 mg (respectively; 1281.7, 640.8, and 320.4 µg/mm³ treatments) (Fig. 13).

**Fig. 13.** Means (±SE) of *C. formosanus* filter paper consumption per surviving termite over 14 days. Food source filter paper was treated with myo-inositol prior to incubation. Collection group C survival data were recorded daily for 2 weeks. Consumption data were recorded at the end of the 2 wk assay. Groups with statistically similar total consumption values bear the same letter (*P* > 0.05). The total consumption *F* and *P* values are 45.99 and 0.0001. The Df values are 3 and 8.
Myo-inositol and phytate (1281.7, 640.8 and 320.4 µg/mm³): Dose-dependent toxicity. At the 1281.7 µg/mm³ treatment level myo-inositol induced significant mortality after d four, phytate did so after d seven (Fig. 14). Both compounds at this dose caused 100% mortality but myo-inositol was more effective at 100% on d six versus phytate on d 10 (Fig. 14). When applied at 640.8 µg/mm³ myo-inositol resulted in significant mortality after d six and 100% morality on d 10. Phytate applied at the same concentration resulted in significant morality after d 12 and 73% morality by d 14 (Fig. 14). At a concentration of 320.4 µg/mm³ myo-inositol again failed to cause statistically significant morality. However, an equivalent dose of phytate showed significant mortality after d 11, ultimately causing 62% morality after 14 d (Fig. 14).

Myo-inositol and phytate (1281.7, 640.8 and 320.4 µg/mm³): Decreased paper consumption. All treatment concentrations examined, for both myo-inositol and phytate, significantly reduced average cellulose consumption per surviving termite when compared to controls (Fig. 15). Individual workers feeding on myo-inositol treated filter paper consumed an average of .062, .05, .116 mg (respectively; 1281.7, 640.8, and 320.4 µg/mm³ treatments) (Fig. 15). Workers feeding on phytate treated filter paper consumed even less than those in the myo-inositol groups. Average consumption per surviving termite in the 1281.7, 640.8, and 320.4 µg/mm³ phytate treatment groups was .029, .045 and .064 mg (respectively) (Fig. 15), the control group being significantly higher at .142 mg (Fig. 15).

Myo-inositol (1281.7, 640.8, 320.4 and 160.2 µg/mm³): Dose-dependent toxicity. In the 14 d assay mortality was not significant in the 160.2 or 320.4 µg/mm³ myo-inositol treatment groups (Fig. 16). Mortality in the 640.8 µg/mm³ treatment group became
significant on d seven and thereafter (Fig. 16). At the highest myo-inositol concentration, 1281.7 µg/mm³, observed morality was statistically distinct from controls after d one and 100% mortality was observed on d six (Fig. 16).

![Graph showing mortality percentage over time for different treatments]

**Fig. 14.** Means (±SE) of C. formosanus percent mortality following treatment of food source filter paper with myo-inositol or phytate in no-choice assays with 20 workers (n=3). Collection group C was used and the data were recorded daily for 14 d. Means followed by the same letter are not significantly different (P > 0.05). The F value for percent mortality is 25.76. P and df values are <0.0001 and 119, 240.
Fig. 15. Means (±SE) of *C. formosanus* filter paper consumption per surviving termite over 14 days. Food source filter paper was treated with *myo*-inositol or phytate prior to incubation. Collection group C survival data were recorded daily for 2 weeks. Consumption data were recorded at the end of the 2 wk assay. Groups with statistically similar total consumption values bear the same letter (*P* > 0.05). The total consumption *F* and *P* values are 38.15 and 0.0001. The Df values are 6 and 14.
**Fig. 16.** Means (±SE) of *C. formosanus* percent mortality following treatment of food source filter paper with *myo*-inositol in no-choice assays with 20 workers (n=3). Collection group E was used and the data were recorded daily for 14 d. Means followed by the same letter are not significantly different (P > 0.05). The F value for percent mortality is 134.81. P and df values are <0.0001 and 74, 150.

**Inter-collection Group Variation in Dose Mortality Assays.** The effects of 1281.7, 640.8 and 320.4 µg/mm\(^3\) treatments, of *myo*-inositol, were examined in three separate mortality bioassays. In the *myo*-inositol 1281.7, 640.8, 320.4 and 16 0.2 µg/mm\(^3\) dose mortality assay, we used termites from collection group E. In the remaining two assays we used collection group C. To examine not only variation between groups collected from the field at different times, but also variation between
groups collected from the same holding container at different times, the termites used in
the 1281.7, 640.8 and 320.4 µg/mm³ myo-inositol dose mortality will be referred to as
collection group C and the termites used in the 1281.7, 640.8 and 320.4 µg/mm³ myo-
inositol and phytate dose mortality assay will be referred to as collection group C’ and
will be treated as separate collection groups. The results of an ANOVA followed by
Tukey’s Studentized range test (Institute 1989) indicated no inter-collection group
variation in resistance, to myo-inositol induced mortality, at the 320.4 µg/mm³ treatment
level. However, at the 640.8 and 1281.7 µg/mm³ treatment levels some variation was
observed. Specifically, at the 640.8 µg/mm³ dose, mortality observed in group C (myo-
inositol 1281.7, 640.8, and 320.4 µg/mm³ dose mortality assay) was significantly
different from groups E (myo-inositol 1281.7, 640.8, 320.4 and 160.2 µg/mm³ dose
mortality assay) and C’ (myo-inositol and phytate 1281.7, 640.8 and 320.4 µg/mm³ dose
mortality assay) on day four, five and six. At the same dose, mortalities observed in all
three groups were different on day seven and the group E (myo-inositol 1281.7, 640.8,
320.4 and 160.2 µg/mm³ dose mortality assay) was different from groups C (myo-
inositol 1281.7, 640.8, and 320.4 µg/mm³ dose mortality assay) and C’ (myo-inositol
and phytate 1281.7, 640.8 and 320.4 µg/mm³ dose mortality assay) on day eight, nine and
10. Additionally, at 1281.7 µg/mm³, mortality in collection group E (myo-inositol 1281.7,
640.8, 320.4 and 160.2 µg/mm³) was significantly different than for both collection
groups C (myo-inositol 1281.7, 640.8, and 320.4 µg/mm³) and C’ (myo-inositol and
phytate 1281.7, 640.8 and 320.4 µg/mm³) on day two and three.
Complementary Log-Log Modeling Of Mortality Data. The results of time-concentration-mortality CLL modeling of C. formosanus are shown in Table 3. The $\beta$ parameter from the maximum likelihood estimation in the CLL model is the slope value ($\beta$). The relationship between time, concentration and mortality using the CLL model for myo-inositol and phytate against C. formosanus is presented with the observed mortality for comparison in Figures 17 and 18 (respectively). CLL modeling for both compounds resulted in a trend of accumulated mortality over time increasing with concentration, indicating the onset of toxicity is concentration dependent. LC$_{50}$ and LC$_{90}$ estimates with 95% confidence intervals for myo-inositol and phytate against C. formosanus are shown in Table 4. The highest concentration evaluated for both compounds was 1281.7 $\mu$g/mm$^3$ (20 mg per 42 mm filter paper), so concentrations represented exceeding this value are from extrapolation. Figures 19 and 20 depict the LT$_{50}$ estimates for myo-inositol and phytate (respectively) against C. formosanus. Estimated LT$_{50}$ values decreased with increasing concentration (Figs. 19 and 20).

When myo-inositol mortality data were pooled and subjected to ANOVA followed by Tukey’s Studentized range test (Institute 1989) mortalities were deemed significantly different from controls after d three and five in the 1281.7 and 640.8 8 $\mu$g/mm$^3$ treatment groups (Fig. 21). In the 320.4 and 160.2 7 $\mu$g/mm$^3$ treatment groups mortalities observed were not significantly distinct from controls (Fig. 21).

Protozoa Quantification. Myo-inositol bioassay one (1281.7 $\mu$g/mm$^3$): Reduced populations. Feeding on filter paper treated with the highest dose examined during dose-response feeding assays, 1281.7 $\mu$g/mm$^3$, of myo-inositol, resulted in significantly reduced total protozoan populations on d three and thereafter (Fig. 22). P.
**grassii** had, a significantly reduced population on d three through 11 (Fig. 22).

Additionally, reduced populations were observed on d four, five and eight, and d four through eight, 11 and 13 for *H. hartmanni* and *S. leidyi*, respectively (Fig. 22).

**Table 3. Parameters used in evaluating CLL modeling of time-concentration mortality for *C. formosanus* workers subjected to *myo*-inositol and phytate.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Df</th>
<th>Scaled Deviance</th>
<th>Mean Deviance</th>
<th>Slope (β)</th>
<th>SE of B</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>myo</em>-inositol</td>
<td>533</td>
<td>1358.4</td>
<td>2.59</td>
<td>.9297</td>
<td>.0842</td>
</tr>
<tr>
<td>phytate</td>
<td>178</td>
<td>355.16</td>
<td>2</td>
<td>1.46</td>
<td>.3413</td>
</tr>
</tbody>
</table>

*Myo*-inositol bioassay two (1281.7 µg/mm³): Reduced populations. In the second assay at 1281.7 µg/mm³ *myo*-inositol protozoan populations were again significantly reduced (Fig. 23), however, less than assay one. Total protozoan populations were found to be significantly reduced on d eight through 11 (Fig. 23). *P. grassii* populations were the most affected, being significantly lower than controls on d four, five, seven, eight, 10 and 11 (Fig. 23). *H. hartmanni* populations were also significantly reduced on d seven, eight, 10 and 11. Although there appeared to be a decline in *S. leidyi* populations, at no point during the 14 d assay were the decreases statistically significant (Fig. 23).

*Phytate bioassay (1281.7 µg/mm³): No significant impact.* A 1281.7 µg/mm³ treatment of phytate failed to significantly reduce any of the three protozoan populations monitored. A general decline in protozoan population was observed in both control and treatment groups (Fig. 24).
Table 4. Estimates of LC50 and LC90 in µg/mm³ with 95% confidence intervals at different periods for *C. formosanus* workers.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time, d</th>
<th>LC50 (95% limits)</th>
<th>Time, d</th>
<th>LC90 (95% limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo-inositol</td>
<td>1</td>
<td>1892807080.0³ (1892805046-1892809113)</td>
<td>1</td>
<td>3706132274.1³ (37061319861-3706132562)</td>
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<tr>
<td></td>
<td>2</td>
<td>1627859.1³ (1627765.6-1627952.6)</td>
<td>2</td>
<td>31873618.1³ (31873504.1-31873732.1)</td>
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<tr>
<td></td>
<td>3</td>
<td>954130.7³ (954042.6-954218.9)</td>
<td>3</td>
<td>18681960.5³ (18681854.2-18632066.9)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8778.3³ (8711-8845.6)</td>
<td>4</td>
<td>171879.4³ (171805.8-171953)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1594.4³ (1529.2-1659.6)</td>
<td>5</td>
<td>31218.1³ (31149.1-31287.1)</td>
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<tr>
<td></td>
<td>6</td>
<td>629.9 (565.2-694.7)</td>
<td>6</td>
<td>12334.4³ (12267.1-12401.7)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>457.3 (392.6-552)</td>
<td>7</td>
<td>8954.1³ (8887.2-9102.9)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>335.8 (271.1-400.4)</td>
<td>8</td>
<td>6574.5³ (6508.1-6641)</td>
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<td>9</td>
<td>288.4 (223.8-353.1)</td>
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<td>5647.3³ (5581-5713.5)</td>
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<td>221.8 (157.1-286.5)</td>
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<td></td>
<td>12</td>
<td>116.9 (52-181.7)</td>
<td>12</td>
<td>3224245.1 (3223666.6-3224823.5)</td>
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<tr>
<td></td>
<td>13</td>
<td>107.4 (42.6-172.3)</td>
<td>13</td>
<td>8954.1³ (8887.2-9102.9)</td>
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<tr>
<td></td>
<td>14</td>
<td>88 (23.1-153)</td>
<td>14</td>
<td>1723.3³ (1658.1-1788.5)</td>
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<td>phytate</td>
<td>1</td>
<td>Confidence interval couldn't be calculated</td>
<td>1</td>
<td>Confidence interval couldn't be calculated</td>
</tr>
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<td>9691371 (9689783.8-9692958.2)</td>
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<td>3</td>
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<td>3224245.1 (3223666.6-3224823.5)</td>
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<td>1069962.4 (1069669.9-1070255)</td>
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<td>65161.5 (65042-65281.1)</td>
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<td>3224245.1 (3223666.6-3224823.5)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1346.7 (1280.7-1412.8)</td>
<td>9</td>
<td>2781.3³ (2715.7-2846.8)</td>
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<tr>
<td></td>
<td>10</td>
<td>705.8 (640.8-770.9)</td>
<td>10</td>
<td>2781.3³ (2715.7-2846.8)</td>
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<tr>
<td></td>
<td>11</td>
<td>490.4 (425.4-553.3)</td>
<td>11</td>
<td>3275.4 (3205.7-3345.1)</td>
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<tr>
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<td>12</td>
<td>272.3 (206.8-337.8)</td>
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<td>1818.9 (1751.9-1885.9)</td>
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<tr>
<td></td>
<td>13</td>
<td>212.5 (146.5-278.6)</td>
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<td>1419.7 (1353.5-1485.8)</td>
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<tr>
<td></td>
<td>14</td>
<td>139.6 (72.1-207)</td>
<td>14</td>
<td>932.4 (867.2-997.6)</td>
</tr>
</tbody>
</table>

³Estimate is extrapolated as the highest concentration used was 1281.7 µg/mm³
Fig. 17. Relationship between observed and CLL model predicted percentage mortality, myo-inositol concentration, and time for *C. formosanus* workers.
Fig. 18. Relationship between observed and CLL model predicted percentage mortality, phytate concentration, and time for *C. formosanus* workers.
Fig. 19. Concentration dependent LT50 estimates for myo-inositol on *C. formosanus* workers.

Fig. 20. Concentration dependent LT50 estimates for phytate on *C. formosanus* workers.
Fig. 21. Means (±SE) of pooled *C. formosanus* percent mortality following treatment of food source filter paper with *myo*-inositol in no-choice assays with 20 workers. Collection groups C and E were used and the data were recorded daily for 14 d. Means followed by the same letter are not significantly different (*P* > 0.05). For controls, 160.2, 320.4, 640.8 and 1281.7 µg/mm³ treatment groups, n values are 18, 3, 9, 9 and 9, respectively. The *F* value for percent mortality is 115.72. *P* and df values are <.0001 and 74, 645.
Fig. 22. Means (±SE) of *C. formosanus* hindgut protozoan counts. The hindgut protozoa of workers from collection group F were enumerated for 14 d while termites were allowed to feed on filter paper treated with 1281.7 µg/mm³ of myo-inositol. Means followed by the same letter are not significantly different (*P* > 0.05). The *F* values for total protozoa, Pseudotrichonympha, Holomastigotoides and Spirotrichonympha are 21.77 (*P* = < 0.0001), 23.12 (*P* = < 0.0001), 7.83 (*P* = < 0.0001) and 12.18 (*P* = < 0.0001), respectively. The df value is 23, 48 for all.
Fig. 23. Means (±SE) of *C. formosanus* hindgut protozoan counts. The hindgut protozoa of workers from collection group F were enumerated daily for 14 d while termites were allowed to feed on filter paper treated with 1281.7 µg/mm³ of myo-inositol. Means followed by the same letter are not significantly different (*P* > 0.05). The *F* values for total protozoa, Pseudotrichonympha, Holomastigotoides and Spirotrichonympha are 10.72 (*P* < 0.0001), 26.71 (*P* < 0.0001), 7.76 (*P* < 0.0001) and 3.69 (*P* < 0.0001), respectively. The df value is 29, 60 for all.
Fig. 24. Means (±SE) of *C. formosanus* hindgut protozoan counts. The hindgut protozoa of workers from collection group F were enumerated daily for 8 d while termites were allowed to feed on filter paper treated with 1281.7 µg/mm³ of phytate. Means followed by the same letter are not significantly different (*P* > 0.05). The *F* values for total protozoa, *Pseudotrichonympha*, *Holomastigotoides* and *Spirotrichonympha* are 7.49 (*P* = < 0.0001), 9.02 (*P* = < 0.0001), 5.66 (*P* = < 0.0001) and 3.28 (*P* = .0013), respectively. The df value is 17, 36 for all.

**Termite Artificial Agar Diet Assays.** *Myo-inositol and D-glucose (40mg/mL agar).* Reduced toxicity. In our first termite artificial agar diet assay *myo*-inositol and *D*-glucose (Fig. 25) were examined. Interestingly, when administered in the absence of a cellulosic food source, *myo*-inositol failed to induce mortality significantly different than was observed in controls supplied *D*-glucose (Fig. 26). Thirty four and 31 % morality was observed after 14 d in *myo*-inositol and *D*-glucose groups, respectively (Fig. 26).
**Fig. 25.** Chemical structure of $D$-glucose.

**Fig. 26.** Means (±SE) of *C. formosanus* percent mortality following feeding on agar containing 40 mg/mL *myo*-inositol or *D*-glucose in no-choice assays with 20 workers (n=3). Collection group B was used and the data were recorded for 14 d. Means followed by the same letter are not significantly different ($P > 0.05$). The $F$ value for percent mortality is 4.84. $P$ and df values are <0.0001 and 29, 270.
Myo-inositol, D-glucose, D-galactose, 2-deoxy-D-galactose (40mg/mL agar) and agar alone. Reduced toxicity. Our second termite artificial agar diet assay was conducted identically to the first with the exception that agar alone, D-galactose (Fig. 27) and 2-deoxy-D-galactose (Fig. 28) groups were added. Myo-inositol, D-galactose and D-glucose diets all failed to induce mortality significantly different from that observed in the agar alone group, all resulting in less than nine % mortality after 14 d (Fig. 29). Specifically, nine, four, one and three % mortality was observed in the D-glucose, D-galactose, myo-inositol and agar alone groups, respectively (Fig. 29). As expected, the 2-deoxy-D-galactose positive control group induced mortality significantly different from that observed in the agar alone group following d 10 and ultimately caused 85 % cumulative mortality (Fig. 29).

Fig. 27. Chemical structure of D-galactose.
Fig. 28. Chemical structure of 2-deoxy-D-galactose.
Fig. 29. Means (±SE) of *C. formosanus* percent mortality following feeding on agar or agar containing 40 mg/mL myo-inositol, D-glucose, D-galactose or 2-deoxy-D-galactose in no-choice assays with 20 workers (n=3). Collection group D was used and the data were recorded daily for 14 d. Means followed by the same letter are not significantly different (*P* > 0.05). The *F* value for percent mortality is 28.11. *P* and df values are <0.0001 and 74, 225.
Red Imported Fire Ant myo-Inositol Toxicity Screen. Myo-inositol and D-sucrose (40 and 10 mg/mL agar). Lack of toxicity. In the first assay red imported fire ants were supplied agar diets containing either D-sucrose (Fig. 30) or myo-inositol for 38 d at concentrations of 40 and 10 mg/mL agar (Fig. 31 A). The mortality observed at both concentrations in the myo-inositol and D-sucrose groups was statistically identical throughout the duration of the study. When administered at 40 mg/mL 63 and 51 % mortality was observed in the D-sucrose and myo-inositol groups, respectively, after the same time period (Fig. 31 A). At the lower concentration of 10 mg/mL 48 and 40 % mortality was observed in the D-sucrose and myo-inositol groups, respectively (Fig. 31 A).

Fig. 30. Chemical structure of D-sucrose.
Fig. 31. Means (±SE) of *S. invicta* percent mortality following feeding on agar containing 40 and 10 mg/mL *myo*-inositol or D-sucrose (A). *S. invicta* represented in B were provided agar containing 40 mg/mL D-glucose or agar with no additional carbon source. The average starting number of ants was 82.7 ± a standard error of 11.9 (n=5). Data were recorded for 38 d (A) and 42 d (B). Means followed by the same letter are not significantly different (*P* > 0.05). The *F* values for percent mortality are 6.98 (A) and 3.83 (B). *P* and df values for A are <0.0001 and 21, 88. *P* and df values for B are <0.0001 and 59, 240.

*D*-glucose (40 mg/mL) and agar alone. In assay two, which was conducted to add additional controls, a comparable 55 and 48 % mortality was observed after 42 d in groups provided either 40 mg/mL D-glucose or agar alone, respectively (Fig. 31 B).

*Myo*-inositol-[2-3H] Radiotracer Study. The resulting aqueous and lipid soluble layers from a Bligh and Dyer extraction (Bligh and Dyer 1959) of whole body homogenates from termites fed *myo*-inositol-[2-3H] (Fig. 32), were assayed for radioactivity. A 100 µL sample of the lipid soluble fraction contained low radiolabel and resulted in 40.8 wide counts per minute (CPM) and 17.6 3H CPM with 9.9 and 15.08 % error, respectively. For comparison, a 100 µL sample of the aqueous layer had 160.9 wide CPM and 131.6 3H CPM with 4.99 and 5.51 % error, respectively. A blank containing only liquid scintillation fluid resulted in 38.9 wide CPM and 14.4 3H CPM with
10.14 and 16.67 % error, respectively, and did not contain an amount of radiolabel significantly different from that observed in the lipid layer. To ensure no low abundance lipid soluble inositol containing compounds were overlooked the lipid layer was concentrated and the radioactivity present was measured again. The concentrated lipid soluble sample did not contain significant radiolabel, so our focus centered on the contents of the aqueous soluble layer. The radioactivity measured in 80 one mL fractions collected from a silica gel column is illustrated in Fig. 33. The chromatograms representing both non-concentrated samples and samples concentrated four fold illustrate a single peak of radioactivity (Fig. 33). All fractions composing a portion of this peak were developed on TLC plates and their Rf values were determined. They were then scraped in fractions and analyzed using LSC. The results of this portion of the experiment are illustrated in Fig. 34 and indicate all radioactivity was localized to a region of the plates consistent with myo-inositol's Rf value. These data support the surprising conclusion that myo-inositol is not biochemically changed following it's consumption by C. formosanus.

Fig. 32. Chemical structure of myo-inositol-[2-^3^H].
Fig. 33. Radioactivity detected in unconcentrated and 4x concentrated aqueous phase silica column fractions of Bligh and Dyer extract of *C. formosanus* homogenate. Twenty workers from collection group A used.
DISCUSSION

Toxicity of *myo*-inositol and phytate to the Formosan Subterranean Termite is reported herein. The most toxic compound examined, *myo*-inositol, significantly increased mortality when applied to food source filter paper discs at 640.8 & 1281.7 µg/mm³ in three independent bioassays (Figs. 12, 14 and 16). In addition to increasing mortality *myo*-inositol also significantly decreased feeding behavior when applied at concentrations ranging from 320.4-1281.7 µg/mm³ (Figs. 13 and 15). The same effect was observed for phytate at identical concentrations (Fig. 15). Phytate also increased mortality, these increases were deemed statistically significant at concentrations ranging from 320.4-1281.7 µg/mm³ (Fig. 14).

*C. Formosanus* termites have been reported to live in the absence of a food source for at least 12 d without significant mortality (Ibrahim et al. 2004), and in our experiments as long as 40 d. So, the fact that *myo*-inositol and phytate decrease
feeding behavior does not provide a plausible explanation for the observed mortality. At the present we are uncertain if the compounds act as feeding deterrents or if the decreased cellulose consumption observed is a symptom of an overall inositol-caused reduction in termite health.

Termites stripped of their protozoan symbionts, by rearing on artificial agar diets, experience significant mortality when switched back to cellulosic food sources (Tanaka et al. 2006a). Therefore, the observation that myo-inositol significantly reduces hindgut protozoan populations (Figs. 22 and 23) introduces the possibility that their toxicity is a secondary effect resulting from decreased symbiont populations. In the case of myo-inositol, this hypothesis is supported by our finding that myo-inositol toxicity is eliminated when it is administered in an agar rather than cellulosic diet (Figs. 26 and 29). The finding that 2-deoxy-D-galactose maintained its toxicity (Fig. 29) when administered in an agar diet provided a positive control and illustrates that tandem administration of toxicants in agar and cellulosic diets may be used to determine if toxicants act directly on termites. Further support is lent to the myo-inositol “death by symbionts” hypothesis by radiotracer studies that show myo-inositol is not chemically changed following its consumption (Figs. 33 and 34) during the toxicity window, which may imply it is not effectively transported across the hindgut membrane. If it is in fact transported it would appear that free myo-inositol, if ingested is not involved in any termite biochemical transformations.

MATERIALS AND METHODS

Insect Collection and Maintenance. Worker C. formosanus were collected from Brechtel Park, New Orleans, Louisiana by methods of Smith et al. (2004). Bait
crates (open-mesh plastic containers) filled with southern yellow pine or spruce-pine-fir sapwood were arranged into a lattice and buried near a *C. formosanus* infested tree and collected after 3 to 9 wk. Termite-containing crates were held in 250-liter plastic garbage cans kept at room temperature (26-28 °C) and 70-80% relative humidity with the original food source. Termites were collected from New Orleans on 1 March 2008 (collection group A, *myo*-inositol radiotracer study); 9 May 2008 (collection group B *myo*-inositol and *D*-glucose artificial agar diet termite assay); 29 May 2008 (collection group C, *myo*-inositol 1281.7, 640.8 and 320.4 µg/mm³ dose mortality assay and *myo*-inositol and phytate 1281.7, 640.8 and 320.4 µg/mm³ dose mortality assay); 20 March 2009 (collection group D, *myo*-inositol, 2-deoxy-*D*-galactose, *D*-glucose and *D*-galactose glucose artificial agar diet termite assay); 29 May 2009 (collection group E, *myo*-inositol 1281.7, 640.8, 320.4 and 160.2 µg/mm³ dose mortality assay) and 11 May 2010 (collection group F, both *myo*-inositol protozoa counting experiments and the phytate protozoa counting experiment). The termite extraction procedure was tapping infested wood sticks into clean plastic containers where they were collected on moistened paper towels.

Red imported fire ants were collected in Baton Rouge, LA near the Louisiana State University campus. Ant mounds were dug up, placed in buckets for temporary transport then transferred to plastic bins in the laboratory. Crickets were provided as food *ad libitum* twice a wk, and water and sugar dishes were filled as needed. Wooden applicators were used as bridges to provide ants access to dishes.
Chemicals. Neutral Red dye, myo-inositol, phytate, myo-inositol-[2-\(^3\)H], 2-deoxy-D-galactose and D-sucrose were obtained from Sigma-Aldrich (St. Louis, MO). D-galactose and D-glucose were obtained from Matheson Coleman and Bell (Cincinnati, OH) and Fisher Scientific (Fair Lawn, NJ), respectively. The chemical structures of myo-inositol, phytate, myo-inositol-[2-\(^3\)H], 2-deoxy-D-galactose, D-sucrose, D-galactose and D-glucose are show in Figs. 10, 11, 32, 28, 30, 27 and 25, respectively.

Dose-Response Feeding Assays. myo-Inositol and phytate were screened for insecticidal activity as follows: Test compounds were applied to 42.5 mm filter papers, in 60 x 15 mm plastic Petri dishes, at the beginning of each experiment, a solution consisting of 1 mg of carbohydrate per 10 µl of distilled water (dH\(_2\)O) was always used. The effects of 2.5 (160.2 µg/mm\(^3\) of filter paper), 5 (320.4 µg/mm\(^3\)), 10 (640.8 µg/mm\(^3\)) and 20 (1281.7 µg/mm\(^3\)) mg of myo-inositol were examined. The same was done for phytate with the exception that 2.5 mg was not tested. Following filter paper treatment dH\(_2\)O was added to bring the total volume of liquid applied to the filter papers to 200 µl and all control filter papers received 200 µL of dH\(_2\)O. To prevent desiccation 100 µL of dH\(_2\)O was applied to filter papers throughout the trials on an as needed basis, approximately every third day. Twenty worker termites were incubated in the dark in a parafilm sealed Petri dish at room temperature for 2 wk. Termite mortality was recorded daily for 14 d in triplicate experiments. An analysis of variance (ANOVA) (SAS ANOVA procedure) followed by Tukey’s Studentized range test was used to evaluate statistical differences among groups (Institute 1989). All mortality data were judged at \(\alpha = 0.05\).

Termites from collection group C were used in both the myo-inositol 1281.7, 640.8 and 320.4 µg/mm\(^3\) dose mortality assay and the myo-inositol and phytate 1281.7,
640.8 and 320.4 µg/mm³ dose mortality assay. Finally, termites from collection group E were used in the myo-inositol 1281.7, 640.8, 320.4 and 160.2 µg/mm³ dose mortality assay.

In dose-mortality experiments where paper consumption data are shown, filter papers were weighed prior to sugar application. After 14 d termites were removed from the dishes and the filter paper from each replicate was cleaned, washed of residual carbohydrate and dried at 100 ºC for 24 h. After drying and standing for 24 h, to allow time for the absorption of atmospheric moisture, filter papers were reweighed to determine consumption. An analysis of variance (ANOVA) (SAS ANOVA procedure) followed by Tukey's Studentized range test was used to evaluate statistical differences among groups (Institute 1989). All consumption data were judged at α = 0.05.

**Complementary Log-Log Modeling.** A serial time-concentration-mortality design was implemented, and conditional mortality probability was estimated using the complementary log-log (CLL) model described by Robertson and Preisler (1992). SAS PROC GENMOD (SAS Institute 1989) was used to obtain maximum likelihood estimates of the conditional response parameters, which were in turn used to estimate cumulative mortality probabilities (Robertson and Preisler 1992). A small positive amount was added to all concentration levels to include controls (Robertson and Preisler 1992). LC$_{50}$ and LC$_{90}$ values with confidence limits were estimated for myo-inositol and phytate using the formulae given by Robertson and Preisler (1992) and LT$_{50}$ values were estimated by linear interpolation (Nowierski et al. 1996, Feng et al. 1998). Additionally, pooled myo-inositol data were subjected to ANOVA followed by Tukey's Studentized range test (SAS Institute 1989). All data were judged at α = 0.05.
Protozoan Counting. The effect of myo-inositol and phytate on C. formosanus hindgut protozoan populations was examined as follows: Twenty mg of myo-inositol or phytate were dissolved in 200 μl of dH2O and applied to filter paper, in a 60 x 15 mm plastic Petri dish, (1281.7 μg/mm³). Control filter paper received 200 μl of dH2O. With 75 worker termites per dish, collection group F were used in both myo-inositol assays and the phytate assay. *Pseudotrichonympha grassii* Koidzumi, *Holomastigotoides hartmanni* Koidzumi and *Spirotrichonympha leidyi* Koidzumi were counted daily for 2 wk as described by Mannesmann (1972) and modified by Maistrello et al. (2002). Hindguts were removed from the posterior ends of three workers and gently macerated in 40 μl of saline solution containing neutral red dye (0.5 ml of 1 % aqueous neutral red solution dissolved in 10 ml saline solution). The number of each protozoan species was determined using a hemocytometer (Bright-line Improved Neubauer, Hausser Scientific, Horsham, PA) under a light microscope. The population of each protozoan species per hindgut ($X_F$) was calculated as: $X_F = (Gn)/(Vx3)$, where $G =$ volume (μl) of solution hindguts dissected in; $n =$ mean of two counts within hemocytometer; $V =$ volume (μl) of area counted. Mean (±SE) $X_F$ values calculated from two replicates were used for graphical comparison of data. Protozoan population data were subjected to ANOVA followed by a Tukey’s Studentized range test (SAS Institute 1989), all data were judged at $\alpha = 0.05$. A square root transformation was applied for data analysis, however untransformed means are reported.

Myo-Inositol Administered in the Absence of Cellulose Feeding Bioassay. Artificial diets were prepared as described by Tanaka et al. (2006b). Briefly, 400 mg/10 mL of selected sugars and 150 mg/10 mL of agar were dissolved in dH₂O, pH 6.8.
Sugar and agar solutions were autoclaved separately. Agar-sugar solutions were poured in 60x15 mm polystyrene petri dishes 5 mm deep, allowed to solidify and then divided into quadrants. **Myo-inositol**, **D-glucose**, 2-deoxy-**D**-galactose and **D**-galactose were all examined as carbon sources in addition to one group that received agar alone. In triplicate experiments, 20 worker termites from collection group B, for the **myo**-inositol and **D**-glucose (40mg/mL agar) assay, or collection group D, for the **myo**-inositol, **D**-glucose, **D**-galactose, 2-deoxy-**D**-galactose (40mg/mL agar) and agar alone assay, were placed in 60x15 mm polystyrene petri dishes and provided with an agar-sugar quadrant to serve as a food and water source. After 14 d termites were removed from the dishes. Mortality data were subjected to ANOVA followed by Tukey’s Studentized range test (Institute 1989). All data were judged at $\alpha = 0.05$.

**Red Imported Fire Ant **myo**-Inositol Toxicity Screen.** **Myo**-inositol was screened for toxicity against the Red Imported Fire Ant. Artificial diets were prepared as described in the former section, with the exception that an additional lower carbon source concentration was included for two of the carbohydrates examined. **Myo**-inositol and **D**-sucrose were administered at both 40 and 10 mg/mL agar whereas **D**-glucose was only provided at 40 mg/mL agar. An additional group was supplied agar alone with no added carbon source. Red imported fire ants were placed in 60x15 mm polystyrene petri dishes and provided an agar or agar-sugar quadrant to serve as a food and water source. Due to the difficulties inherent in handling ants, a fixed number of ants were not added to each dish. Dishes were instead placed in an enclosure containing ants & quickly sealed once they appeared full. Significant post-transfer mortality was observed within 24 h of transfer, ants that died within this period were not included in the trials.
The average starting number of ants was 82.7 ± a standard error of 11.9. Ants in the myo-inositol and D-sucrose assay were monitored intermittently and mortality data were collected for 38 d. Ants in the D-glucose and agar without an additional carbon source assay were monitored intermittently and mortality data were collected for 42 d. Five replicates were included in each assay and mortality data were subjected to ANOVA followed by Tukey’s Studentized range test (Institute 1989). All data were judged at α = 0.05.

**Myo-inositol Radiotracer Study.** Food source filter paper was treated with 25 μL of a cold 555 mM aqueous myo-inositol solution and 10 μL of a 500 nM aqueous myo-inositol-[2-3H] solution, specific activity 10-20 Ci/mM. Twenty worker termites, from collection group A, were allowed to feed on treated paper for 2 d and frozen. Frozen termites were homogenized and a Bligh and Dyer (Bligh and Dyer 1959) extraction was performed. Aqueous and lipid fractions were concentrated and liquid scintillation counting (LSC) was used to measure radioactivity. The aqueous soluble fraction was fractionated with a silica column (1 cm diameter, 8 cm length) using 4:1 acetonitrile: dH2O as the mobile phase. Eighty 1 mL fractions were collected and LSC was used to evaluate their radioactivity. Fractions found to be radioactive were separated with silica thin layer chromatography using 4:1 acetonitrile:dH2O as the mobile phase. Chromatograms were visualized with iodine vapor and Rf values were compared with myo-inositol standards. The fractions were subsequently scraped from the plates with a razor and their radioactivity was measured using LSC.
REFERENCES


Spink, W. T. 1967. The Formosan subterranean termite in Louisiana., Louisiana State University Circ. 89. Louisiana State University, Baton Rouge, LA.


CHAPTER FOUR

CONCLUSIONS

D-psicose, D-tagatose, D-arabinose, D-lyxose, L-lyxose, D-allose, D-arabitol, L-arabitol, L-Ribose, D-xylitol, L-galactose, D-threitol, DL-threitol, L-xylose, 2-deoxy-D-galactose, 2-deoxy-D-glucose, myo-inositol and phytate were screened for insecticidal activity against *C. formosanus*. From our initial screening experiments D-psicose, D-arabinose, D-lyxose, L-lyxose, D-threitol, 2-deoxy-D-galactose, 2-deoxy-D-glucose, myo-inositol and phytate were selected for further investigation. Of these compounds 2-deoxy-D-galactose, myo-inositol and phytate were identified as potential termiticides.

When the LC$_{50}$ values for each of the three compounds are compared it is clear that myo-inositol is the most toxic, followed by 2-deoxy-D-galactose and phytate, with phytate being the least toxic. Both myo-inositol and 2-deoxy-D-galactose significantly reduce *C. formosanus* hindgut populations of *Pseudotrichonympha grassii* Koidzumi, *Holomastigotoides hartmanni* Koidzumi and *Spirotrichonympha leidyi* Koidzumi. The fact that myo-inositol is only toxic when administered with a cellulosic food source and is not chemically altered following its ingestion points to a mechanism of action involving the disruption of proper digestion. Additionally the finding that 2-deoxy-D-galactose continues to causes termite mortality regardless of the presence of a cellulosic food source implies that, while its ingestion causes a decline in protozoan populations, its mechanism of action targets the termite directly.
These findings support the conclusion that using natural compounds, such as carbohydrates and cyclitols, to selectively kill Formosan termites with little or no environmental impact is a valid possibility. Future delayed-effect carbohydrate and cyclitol termite population control strategies may target termites directly or aim to eliminate hindgut symbionts while cellulose is being consumed, indirectly causing death. With either strategy because 2-deoxy-\(\text{D}\)-galactose, \textit{myo}-inositol and phytate all take approximately one week to induce mortality, if added to baits, the compounds should allow time after bait discovery for an entire colony to be affected.
Appendix A1. The chemical structure of rare sugars in the library screened for activity against *C. formosanus*. 
Appendix A2. Mean number of dead *C. formosanus* following treatment of food source filter paper with 5 mg *D*-tagatose, *D*-xylitol or 2-deoxy-*D*-glucose in no-choice assays with 20 workers (n=3). The data were recorded daily for 14 d. At the end of the 14 d bioassay the mortality observed in the 2-deoxy-*D*-glucose treated group was found to be statistically different than controls, as judged by non-overlapping standard errors.
Appendix A3. Mean number of dead *C. formosanus* following treatment of food source filter paper with 5 mg *L*-arabitol, *L*-lyxose, *L*-xylose, *DL*-threitol or *D*-allose in no-choice assays with 20 workers (n=3). The data were recorded daily for 14 d. At the end of the 14 d bioassay no groups were deemed significantly different than controls.
Appendix A4. Mean number of dead *C. formosanus* following treatment of food source filter paper with 5 mg of \(L\)-lyxose, \(D\)-arabitol, \(L\)-galactose or \(L\)-ribose in no-choice assays with 20 workers (n=3). The data were recorded daily for 14 d. At the end of the 14 d bioassay the mortality observed in the \(L\)-lyxose treated group was found to be statistically different than controls, as judged by non-overlapping standard errors.
Appendix A5. Mean number of dead *C. formosanus* following treatment of food source filter paper with 5 mg of \(D\)-psicose, \(D\)-arabinose, \(D\)-threitol, 2-deoxy-\(D\)-galactose or \(D\)-lyxose in no-choice assays with 20 workers (n=3). The data were recorded daily for 14 d. At the end of the 14 d bioassay the mortalities observed in the 2-deoxy-\(D\)-galactose, \(D\)-threitol and \(D\)-Psicose treated groups were found to be statistically different than controls, as judged by non-overlapping standard errors.
Appendix A6. Mean number of dead *C. formosanus* following treatment of food source filter paper with 2.5, 5 or 10 mg of *L*-lyxose in no-choice assays with 20 workers (n=3). The data were recorded daily for 14 d. At the end of the 14 d bioassay the mortality observed in the 10 mg treatment group was found to be statistically different than controls, as judged by non-overlapping standard errors.
Appendix A7. Mean number of dead *C. formosanus* following treatment of food source filter paper with 5, 10 or 20 mg of \( L \)-lyxose in no-choice assays with 20 workers (n=3). The data were recorded daily for 14 d. At the end of the 14 d bioassay the mortalities observed in the 10 and 20 mg treatment groups were found to be statistically different than controls, as judged by non-overlapping standard errors.
Appendix A8. Mean number of dead *C. formosanus* following treatment of food source filter paper with 2.5, 5 or 10 mg of *D*-threitol in no-choice assays with 20 workers (n=3). The data were recorded daily for 14 d. At the end of the 14 d bioassay the mortality observed in the 10 mg treatment group was found to be statistically different than controls, as judged by non-overlapping standard errors.
Appendix A9. Mean number of dead *C. formosanus* following treatment of food source filter paper with 2.5, 5 or 10 mg of *D*-arabinose in no-choice assays with 20 workers (n=3). The data were recorded daily for 14 d. At the end of the 14 d bioassay the mortality observed in the 10 mg treatment group was found to be statistically different than controls, as judged by non-overlapping standard errors.
Appendix A10. Mean number of dead *C. formosanus* following treatment of food source filter paper with 2.5, 5 or 10 mg of 2-deoxy-\(\text{D}\)-glucose in no-choice assays with 20 workers (n=3). The data were recorded daily for 14 d. At the end of the 14 d bioassay no groups were deemed significantly different than controls.
Appendix A11. Means (±SE) of the percent change in mass of filter papers fed on by *C. formosanus* over 14 days. Food source filter paper was treated with L-lyxose prior to incubation. Filter paper mass data were recorded at the beginning and the end of the 2 wk assay.
Appendix A12. Means (±SE) of the percent change in mass of filter papers fed on by C. formosanus over 14 days. Food source filter paper was treated with D-threitol prior to incubation. Filter paper mass data were recorded at the beginning and the end of the 2 wk assay.
Appendix A13. Means (±SE) of the percent change in mass of filter papers fed on by *C. formosanus* over 14 days. Food source filter paper was treated with *D*-arabinose prior to incubation. Filter paper mass data were recorded at the beginning and the end of the 2 wk assay.
Appendix A14. Means (±SE) of the percent change in mass of filter papers fed on by *C. formosanus* over 14 days. Food source filter paper was treated with 2-deoxy-D-glucose prior to incubation. Filter paper mass data were recorded at the beginning and the end of the 2 wk assay.
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Lucas James Veillon was born in Lake Charles, Louisiana, in 1981. He attended high school in Clinton, Indiana, where he graduated from South Vermillion High School in 1999. He then moved to Baton Rouge, Louisiana, where he received his bachelor degree from Louisiana State University in 2003. Lucas joined the lab of Dr. Roger Laine in the spring of 2006. He began a post-doctoral appointment, with all but dissertation status, in the fall of 2011 at the Complex Carbohydrate Research Center at the University of Georgia. He defended his dissertation October 27, 2011 and will be graduating December 16, 2011. Lucas plans to continue his work at the University of Georgia where he will conduct research on the characterization of biologically relevant carbohydrates.