Subterranean termite, *Reticulitermes flavipes*, colony behavior and population dynamic changes under conditions of exposure to chitin synthesis inhibitor bait

by

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Abstract

This thesis entitled, “Subterranean termite, *Reticulitermes flavipes*, colony behavior and population dynamic changes under conditions of exposure to chitin synthesis inhibitor bait”, explores key components involved in termiticide baiting success on a colony scale. My thesis incorporates a literature review on subterranean termites, *Reticulitermes flavipes*, population dynamics, life system requirements, as well as current termiticide control methods. The literature review is followed by my two research experiments, “Impacts of Nile Blue A Internal-Stain-Marking on Eastern subterranean termite, *Reticulitermes flavipe*”, and “Video Analysis of Termite Colony, *Reticulitermes flavipes*, Throughout Exposure to Trelona Termiticide Bait”. The first experiment investigates the potential for using biological stain, Nile Blue A, as a marking tool for colony studies involving *Reticulitermes flavipes* and chitin synthesis inhibitor bait products; While the second study analyzes *Reticulitermes flavipes* colonies under video surveillance while being exposed to Trelona (AI: Novaluron) termiticide bait and Nile Blue A biological stain. Many behaviors and observations critical to termiticide baiting success were documented and recorded for the first time at a colony level. My thesis explores and expands our knowledge of the specific interactions involved between subterranean termites and termiticide baits, through biological staining and contemporary video technology.

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CHAPTER 1

Introduction and Literature Review

Subterranean Termites and Humans

Termites are multifaceted eusocial insects with unique and diverse caste systems. One of these facets is the ability to consume and break down cellulose as a food source. Termites can break down cellulose by virtue of bacteria or in some cases protozoa in their digestive system along with their digestive enzymes (Slaytor 1992). Unlike cattle, sheep, or other livestock, termites do not get their energy from the cellulose in grasses, but instead from trees or woody substances. This is problematic for humans because many of the various structures we build and reside in are comprised of or incorporate woody plant materials. Termites are not a potential threat to human health. They are not known to carry any disease harmful to humans or pets. They rarely bite /sting humans. However, they can negatively impact human welfare by causing severe damage to unprotected timber structures, homes, underground cables, earthen dam, sand much more (Ghaly 2011).

In 2010, it was estimated the global economic damages of termites was approximately 40 billion US dollars (Su 2019). In the U.S., the structural pest control market reached $9.359 billion in 2019, a 4.3% increase from the $8.971 billion measured in 2018 (PCT, 2020). To control termites, homeowners spend upwards of $2 billion each year, as reported by the EPA. Currently scientist have documented over 3,000 species of termites, and 79 of those are characterized as destructive pests (Su 2019). These 79 species are comprised of primarily subterranean termites and dry wood termites; ~84% of those pests are of the subterranean termites.

The eastern subterranean termite, *Reticulitermes flavipes* (Kollar), the subject of this thesis, is a species of subterranean termites that are widespread throughout the eastern United States. They perform important ecological roles such as wood decomposition, nitrogen fixation, and also engage in the aforementioned economic damage as a pest organism (Vargo 2000). Termites are universally understood to be eusocial insects with a complex caste system. *Reticulitermes flavipes* is a member of the family Rhinotermitidae which is considered to be a very evolutionary derived termite family with complex reproductive systems and lifestyles (Inward 2007).

External Anatomy & Caste Structure

There are discernable physical differences among subterranean caste and *R. flavipes*, is no exception. *R. flavipes* colonies are comprised of castes which include workers, soldiers, nymphs, pre soldiers, larvae, and various forms of reproductive (Hu, 2011a). Of the various castes in a colony, workers are the most abundant comprising of 84.0% of the total population, while larvae comprised 8.7%, nymphs at 5.0%, adult soldiers at 2.1%, while pre-soldiers along with neotenic reproductives represent approximately 0.1% of the total colony population (Howard 1981). I will next outline the roles of these castes.

Workers are considered members of the caste who forage for food, feed colony members such as reproductives, soldiers, and larvae; they; build tunnels and nest networks, as well as partake in social interactions such as grooming and care for eggs.

Soldiers are larger in size than workers. They have heavy sclerotized and modified heads and guard and protect colony members and must be fed by workers as they cannot feed themselves. Alates are winged adult termites which act as reproductives who initiate and

propagate colonies as future queens and kings (Krishna 1989). Nymph worker termites may develop into presoldiers which later molt into soldiers, remain workers, or transform into reproductive caste members called ergatoids (Hu 2011a). Ergatoids are apterous worker-like secondary reproductives or apterous neotenic reproductives. Pre-soldiers, the caste before soldiers, have a soldier-like head and must also be fed by workers, however the head is unpigmented and unsclerotized. *Reticulitermes flavipes* soldiers have a caste prevalence ratio of 2% of the total colony population (Howard 1980).

It is at the second molt of Rhinotermitidae termites that larvae may differentiate down the wingless worker pathway, or the wing-pad nymph pathway as seen in Figure 1 (Hayashi et al. 2007). The wing-pad nymph pathway is identified as individuals with wing-pads who develop into either imagoes which are alates with wings and eyes who can disperse and initiate newfound colonies, or the wingpad individuals develop into nymphoids also referred to as brachypterous secondary reproductives/ brachypterous neotenic reproductives (Hu 2011a). Nymphoids have no eyes and primitive wing-buds. Nymphoids are unable to fly and subsequently do not disperse. Their colonial role is to replace or supplement the primary reproductives. The loss of primary reproductives can stimulate one or numerous nymphs to transform into neotenic substitutes however, the mechanisms involved in influencing differentiation of neotenics remains unknown (Myles 1999).

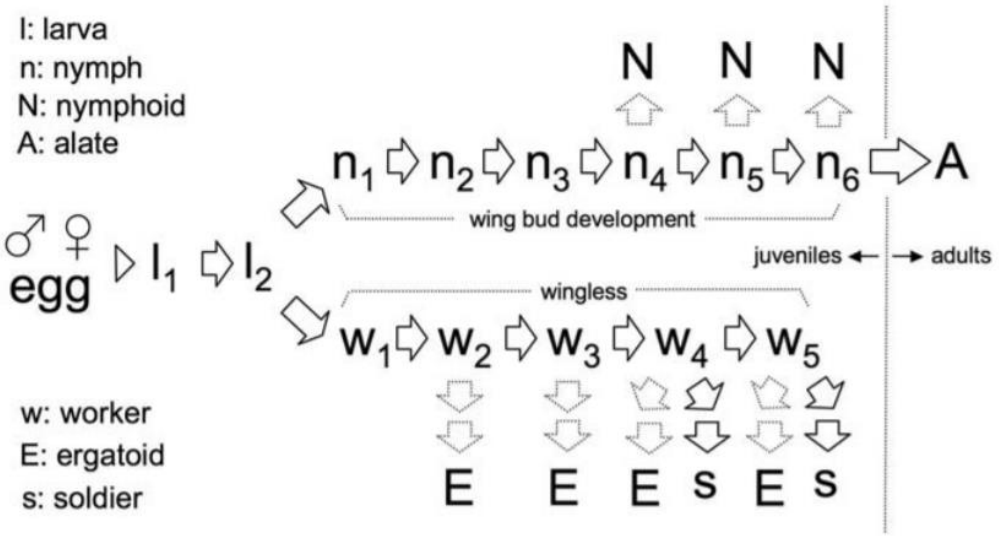

Figure 1: Caste developmental pathways of *Reticulitermes* spp. (Hayashi et al. 2007)

There has been a gene sequence coding identified which codes for a hexamerin protein which may be involved in the differentiation process in nymphs to adult caste members along with juvenile hormone and ecdysone, as the hexamerin protein exemplifies higher expression in nymphs than adult caste members (Hu 2011a). Along with genes which code for adult pathway expression, *R. flavipes*, also exhibit phenotypic plasticity by which one gene, encoding for cuticular hydrocarbon combination vary among differentiated caste members (Howard 1978). The aforementioned study demonstrated soldiers and alates have higher abundances of cuticular hydrocarbons than worker termites which is hypothesized to be due to soldiers and alates requiring greater barriers against water loss allotted to environmental exposure variances. Each caste member and their associated role is vital to the success and continued survival of the colony.

Population Dynamics & Environmental Relationship

Assessing the population dynamics of subterranean termites is not simple. The subterranean nature of these termites makes studying and estimating exact population dynamics [relationships amongst caste members within a population (colony)] and behaviors difficult as the majority of the colonies' actions and life occurrences occur beneath the soil. When discussing the population dynamics of *R. flavipes*, it is important to discuss the reproductive strategy of subterranean termites. It is also important to assess the facets of their caste biology and intra-relationships to draw appropriate conclusions. R-selection and K-selection are the primary reproductive strategies. Subterranean termites are not completely one strategy or the other, however share characteristics from both R-selection and K-selection. Termites have high populations and reproductive growth rates in accordance with R-selection, however, they exhibit brood and maternal care as dictated by K-selective strategists. Researchers have attempted to identify the lifespan of the genus *Reticulitermes*, however estimates vary by study. Some have estimated the maximum lifespan of a queen *R. hesperus* (Banks) at 30 years, while the maximum lifespan of *R. santonesis* at ~ 7 years (Keller 1998). Due to the nature of subterranean termites especially high fecundity, cryptism, and the use of numerous reproductives in the event of primary reproductive death, it is difficult to truly determine lifespans of the castes. In terms of timing of reproduction, *R. flavipes* in conjunction with other termite species would be considered iteroparity as reproduction occurs continuously until the eventual collapse of a colony. As such, examination of whole colonies may be a better gauge of population lifespan and health as termites are eusocial and rely upon each other for continued colony survival. *R. flavipes* for example have been studied in laboratory colonies exceeding 11-years old (Long 2005).

Reticulitermes flavipes colonies show great variation in size as colonies can range from new primary reproductives just starting a colony to millions of individuals. One study found six

R. flavipes colonies to have an estimated mean population size of 244,445 termite individuals (Howard 1982). Another study used mark-release-recapture methods to deduce foraging populations up to 3.2 million individuals (Grace et al. 1989). Colony sizes this large are evidence supporting the reproductive progeny success of *R. flavipes* unique and complex reproductive systems, behavioral interactions, and foraging methods. Using 16S rRNA mitochondrial markers (Austin 2005) the geographic distribution of *R. flavipes* has been identified from North America down to South America, Germany, as well as France. The dynamic geographic distribution of species range is a testament to the survival success this isopteran species possess.

Abiotic Factors

Abiotic factors are critical to the success of *R. flavipes*, because termites are ectothermic, temperature is vital to subterranean termite survival. Drastic changes or fluctuations in temperature can result in either optimal or dreadful conditions for the termites, and consequently affect their behaviors, development and survival. One study feeding and survival of *R. flavipes* noted the highest temperature ranges for long term of survival is between 31.5º and 33ºC (Smythe and Williams 1972). Another study assessed the critical upper lethal limit and critical lower lethal limit of *R. flavipes* and found the upper lethal limit to be between 46.9ºC and 48.3°C, and the critical thermal minimum to be between 1.0ºC and 4.9°C (Hu and Appel 2004). Termites like other insects are considered ectotherms as they are cannot regulate their body temperature and are effected by stationary or ambient temperatures. Due to their reliance on external temperatures, behaviors and movements are affected by local temperatures. A study examined behavioral responses of two subterranean termite species, *R. flavipes* and *Coptorermes formosanus* to chilling ground-surface temperatures, and reported that both termite species moved downward from chilling area to stay in lower warmer areas to avoid encountering

harmful freezing (Hu and Song 2007). Another study assessing termites' ability to evacuate disturbances in soil at varying temperatures found that termites abandon tunnels faster at warmer temperatures and in greater frequency after disturbance than at colder temperatures (Schwinghammer 2006). Feeding may also be affected as temperatures change seasonally causing behavioral responses. It has been found that *R. flavipes*'s feeding rate was greatest in the summer and lowest in the winter (Harahap et al. 2005). Harahap also found that R. *flavipes* did not feed when humidly levels were below 40%.

Additionally, soil quality is an important abiotic factor influencing subterranean termite success. Soil quality is important because subterranean termites require soil substrates to make their tunnel networks that connect their nests and cellulosic food resources. Subterranean termites require moist soils as dry soils can lead to desiccation. Soil type is crucial in tunnel formation and particle size has a direct correlation to tunnel construction (Cornelius and Osbrink 2010). Soil particle size as well as soil humidity directly affects the soil particles movability by the termites as some particles may be easier or harder to move given their size and moisture levels. Soil composition may affect chemical signaling or pheromone trail distances given the chemical properties the soil particles possess which may be more or less conducive to particular microorganisms or bacteria residing within the soil.

Abiotic factors are also crucial to the development and foraging of subterranean termites. Temperature and humidity play a large roll in not only termite biology, but in termite movement and tunnel behaviors. Humidity and soil quality is also essential in understanding the relationship between termites and how they forage. Other abiotic factors such as wind and light can contribute to colony success. Wind patterns possess the ability to disperse reproductive alates over geographic locations, while light is commonly understood to be a stressor to subterranean

termites as they actively avoid light if exposed. It is important to assess abiotic factors when discussing termite survival as termites are small and can experience numerous microclimates within a relatively small area.

Evolution and Coevolution

Subterranean termites, including the species *R. flavipes*, are eusocial organisms which have evolved to include an expansive caste network, dynamic foraging strategies, evolutionary dependent obligate endosymbionts, as well as many other evolutionary biological and behavioral traits and characteristics. For example all termites exhibit cooperative brood care, by which colony members collectively aid in nymphal and larval care, is believed to have arisen when termites evolved from wood dwelling organisms to foraging organisms, and as such cooperative brood care aided increased defenses which are believed to be the initial reason for termite brood care evolutionarily (Korb et al. 2012). The ability to cooperatively raise brood despite the brood not being one's own brood is a fundamental characteristic of eusocial insects and is crucial to the success and continued growth of a colony. A colony would collapse quickly if no members of the colony aided in nymphal and larval care.

Eusocial organisms such as subterranean termites must also be able to differentiate between and identify their respective colony kings and queens in order to feed them, groom them, and ensure continued colony success. A study aimed at expanding the evolutionary understanding of *R. flavipes* social interactions discovered the hydrocarbon heneicosane and identified it as a royal recognition pheromone. This pheromone is present in both kings and queens of *R. flavipes*, with analysis dating the royal pheromone evolution to approximately 150 million years ago (Funaro 2018). This research showed that *R. flavipes* have been incorporating social structure through reproductive pheromone signaling for millions of years thus

exemplifying how fundamental eusociality is within this termite species while also indicating clear delineations between caste members and reproductives.

Coevolution is the process by which two or more species influence each other evolutionarily over time. The clearest evidence of coevolution in termites is in relation to their gut endosymbionts which have coevolved with them to be obligate endosymbionts. Evidence supporting this includes that termites are unable to feed themselves without the aid of their gut endosymbionts that breaks down the lignocellulosic materials. A study, using 16S rRNA, examined the relationship between gut endosymbionts and castes and discovered that castes of *R. flavipes* vary in core gut endosymbionts as well as the abundance of those endosymbionts (Benjamino 2016). The results of this study are useful in that they reinforce that caste members have different colony roles and as such, may not need to contain the same gut endosymbionts or same amount of gut symbionts, considering workers jobs are to collect nutritional resources and feed/take care of the colony members, compared to alates whose primary role is related to reproduction.

Microbe/Endosymbiont

R. flavipes in conjunction with other termite species rely on a vast array of gut endosymbionts to aid in the digestion and acquisition of essential nutrients and sustenance. Aside from unique caste systems and reproductive delineations, beneficial gut endosymbionts are another primary component in relation to the complexness of subterranean termites. These gut microorganisms are considered obligate endosymbionts as termites rely on them to digest and break down lignocellulose and cellulosic materials as their primary food source (Breznak 1994). The various gut endosymbionts are intracellular mutualists as both the termite and the endosymbiont benefit from one another. The endosymbiont is being provided shelter and a

nutrition source, while the termite is receiving broken down cellulosic materials which it can then utilize for energy and sustenance.

As a result of gut endosymbionts being responsible for primary termite nutritional uptake, termites are mostly reliant upon their gut endosymbionts to survive. As such, termites pass down their gut endosymbionts and micro-organismal biome both horizontally and vertically among colony nest mates via social behaviors such as trophallaxis and grooming practices (Ohkuma 2010). The passing-down and sharing of gut endosymbionts within a termite colony ensures caste mates and colony members obtain the necessary gut microbiome which aids in total colony fitness as members possess the important endosymbionts. As such, it is important that once a termite obtains a gut endosymbiont, the endosymbiont attach and survive within the termite gut. The protozoan *Pyrosonympha vertens* for example attaches to the paunch epithelium within *R. flavipes* gut via attachment organelle (Breznak 1977). Breznak also identified 7 bacterial morphotypes within *R. flavipes* paunch. Gene sequencing and 16S rRNA analysis have been performed to identify a range of endosymbiotic ribotypes from within the *R. flavipes* termite gut. Under the domain Bacteria the following were found, *Proteobacteria*, *Spirochaetes*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Endomicrobia* (Fisher 2007). While studies attempt to identify and classify the protozoan or bacteria within *R. flavipes* gut, much is still unknown about the exact functions of the microorganisms residing in the termite gut as most of the protozoans cannot be cultured (Radek 1999).

Due to the inability to culture many of the endosymbionts within the termite gut, researchers attempting to determine microorganism functions must be creative with their approach. A study performed by Hu et al. (2001) assessed *R. flavipes* gut endosymbiont functions by using starvation tests. After a 40-day starvation they found complete elimination of

5 microorganism species within the termite gut, *Trichonympha agilis*, *Pyrsonympha vertens, and P. major, D. gracilis,* and *Holomastigotes elongatum*, a reduction in *Dinenympha fimbriata, and Spironympha kofoidi*, but no effect on *Trichomitus trypanoides* and *Spirotrichonympha flagellate* (Hu, et al 2011). They considered *T. agilis* to be the most important cellulosic species critical for termite survival and nutrition. Being an important location for cellulosic degradation and nutrient uptake, the termite gut is equally important in producing acetate metabolites as a fermentation product of gut endosymbionts, involved in acetogenic pathways and anoxic fermentation processes vital for termite survival (Tholen 1997). Endosymbionts are dynamic within the termite gut and are crucial to termite survival biological processes.

Behavioral Traits

Reticulitermes flavipes performs a variety of social behaviors and interactions among colony members. Trophallaxis, analaxis and coprophagy are key feeding mechanisms since they are important social behaviors that nest mates perform to exchange nutrients or other microorganisms. Trophallaxis is believed to have arisen in termites because it incorporates microbial, nutritional, and social environments which can affect developmental trajectories and eusocial-ability (Nalepa 2015). Trophallaxis is a very common practice among *R. flavipes*. One study examined trophallaxis via isotope transfer and found *R. flavipes* transferred isotope material within hours of initial consumption (Suárez 2000). The ability of workers to transfer consumed materials to other colony member quickly is a double-edged sword depending on the nature of the consumed material (Mirobito and Rosengaus 2016). Workers are responsible to transfer nutrients to feed those who are unable to do so themselves such as colony reproductive, and larvae who are basically confined on and around the nest site. Termite baiting technology

utilizes trophallaxis behavior for termites to transfer consumed bait toxicants to colony-mates and achieves control efficacy (Lewis and Forschler 2016).

Subterranean termites are unique in their methodology of food collection and foraging. As mentioned previously, subterranean termites live cryptically and spend most of their time under the soil surface. As such, their foraging methodology involves creating tunneling networks from the colony sites throughout the soil to cellulosic food resources such as trees, homes with wooden components, woody mulches, etc. Subterranean termites tunnel outwards from their nest until they reach a suitable cellulosic resource, once there, that resource becomes the new center or satellite nests for outwards foraging to locate more cellulosic food resources (CamporaGrace 2000). Even when the termites reach a food source, they continue to forage outwards to ensure continued food resources despite having already located a suitable cellulosic resource. Some species within Rhinotermitidae such as the Formosan subterranean termite, *C. formosanus,* can modify their tunnel sizes based on available food size. These termites invest more in primary tunnels leading to quality food resources by making them larger and more robust, while leaving secondary search tunnels smaller and less developed (Hedlund 1999). Behaviors such as this exemplify the complexity of termite behavior.

Termite Control

Subterranean termite management and control can be accomplished through a variety of methods usually involving the poisoning of termites. Primarily subterranean termite control is carried out via the application of liquid termiticide, or termiticidal bait product. Liquid termiticides are soil insecticides applied under or surrounding a structure to create a treated zone against subterranean termites (Potter and Hillery 2002). Liquid termiticides can come in repellent or non-repellent forms. Organophosphates and pyrethroid termiticides are considered repellant

termiticides as they kill termites quickly upon contact and as such are often used as barrier termiticides (Hu 2011b). Some liquid termiticides are designed similar to bait products, to be non-repellent and slow-acting, meaning they are toxic but do not deter termites from interacting with the toxicants, and show delayed activity to allow exposed termites to share the toxicant amongst colony members (Thorne 2001, Ibrahim et al. 2003, Shelton and Grace 2003, Tomalski 2004). Bait containing Fipronil is considered fast acting insecticides, as termite mortality occurs quickly within days (Fei and Henderson 2005, Remmen and Su 2005, Chouvec 2018).

Termite bait products the primary subject of this thesis have been used commercially since the 1990s and have increased in popularity among pest control professionals due to their unique mode of action, versatility, and effective results (Su 2002). Termite baiting products are typically distributed in a field setting within in-ground or above-ground bait stations. Bait stations are containers which hold the toxic bait product and are subsequently placed into the soil at target sites. It is common for pest management technicians to pre-bait initially with non-active [no insecticide chemical, just cellulosic matrix] bait tablets to examine if there is termite activity before installing active [insecticide infused bait matrix] bait tablets to eliminate termites. Active termite bait products typically use a chitin-synthesis inhibitor as the active ingredient (Su 1993, Su 1996, Grace 1996, Su 2005, Evans 2015). Chitin-synthesis inhibitors (CSI's) disrupt the termite molting process which results in malformed chitin leading to death (Su 1993, Evans 2015, Su 2019). The CSI's used in termite baits are designed to be slow acting and non-repellent in order to facilitate the uptake of the active ingredient by termites during feeding and subsequent spreading of the active ingredient to colony members through trophallaxis, grooming, and other common social behaviors (Su 1993, Grace 1996, Evans 2006, Evans 2015, Su 2019).

It is the exploitation of the social behaviors of termites which aids in the successful elimination of the treated termite colony. The social interactions between termites directly facilitate the movement of the toxic active ingredient among colony members. Thus a non-repellent slow acting active ingredient is crucial in uptake and exchange of the active ingredient. Numerous studies have examined the non-repellency of bait products and demonstrated them to indeed be non-repellent (Su andScheffrahn 1993, Su 2005, Su 2009, Gautam 2014, Chouvenc 2018). It is also claimed that bait products are dose-independent, meaning there is no minimum lethal dose required for colony elimination (Su 1995). Bait efficacy studies have also demonstrated the success of bait products at eliminating termite colonies (Su 1998, Getty 2000, Evans 2015, Chouvenc 2017, Chouvenc 2018). Many bait efficacy studies have been performed using mark-release-recapture methods to identify the success of the bait product in question (Su 1993, Grace 1996, Evans 2001, Crossland 2006, Thoms 2009, Su 2019). One issue with this type of study is that it does not provide the exact time of colony death, only a relative population estimate. Additionally, mark-release-recapture experiments evaluate the efficacy of the bait product through evaluation of apparent mortality given termites recovered, and do not evaluate efficacy based on termite behavioral interactions or colony interactions with the bait. Due to this, complete understanding of the exact mechanisms and efficacy behind termite bait products are still lacking. Furthermore, a comprehensive understanding of the mechanisms and exchanges involved in interactions between bait products and termite colonies as a whole is still incomplete.

In summary, subterranean termites are important social insects which act both as natural decomposers as well as insect pests. The eastern subterranean termite, *R. flavipes*, is one such species which possess a complex biology involving endosymbiont relationships, dynamic social colony level interactions, a distinctive caste structure, and requires multiple abiotic and biotic

inputs for colony and progeny success. *R. flavipes* is considered a pest in much of the world with great potential for causing economic losses due to their ability to break down and digest woody materials in man-made structures. Because of it pest status, there are many control and management strategies available to combat *R. flavipes* and subterranean termites like them. These control methods utilize our available knowledge of subterranean termite biology and behavior to increase the efficacy of the control products applied. The effects of termiticide bait products, while highly effective and popular, are not yet completely understood. The mechanisms and interactions between termiticide bait products and subterranean termites are still theorized despite much evidences supporting their efficacy. This is due to the cryptic nature of these termites, as well as the types of testing protocols which have been done. Novel termiticide bait testing and total colony examination may help fill in the gaps present relating to termiticide bait products and termites.

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CHAPTER 2

Impacts of Nile Blue A Internal-Stain-Marking on Eastern subterranean termite, *Reticulitermes flavipes*

Abstract: The dye marker Nile Blue A was tested as an effective and suitable internal-making method for studying behavioral effects and efficiency of a termite bait against the Eastern subterranean termite, *Reticulitermes flavipes* (Kollar). The sensibility of termites to the dye marker was examined by measuring the survival, chance in coloration, and transferability (lateral and vertical) of *R. flavipes* according to the concentrations and feeding period of feed treated with Nile Blue A solution. Within 3 weeks of food ingestion, termites showed no significantly different in mortality among groups fed feed containing 0%, 0.1% 0.2%, or 0.3% concentrations of Nile Blue A. At week 4 and 5, termites of 0.3% Nile Blue A concentration had significantly greater mortality (about 35%) than other concentration groups (between 10% - 20%). As the dye concentration increased, termites were stained faster and darker, until week 4 when termite groups of 0.1% and 0.2%, except for 0.3% concentration, displayed similar color. Nile Blue A was not transferred noticeably from stained to unstained termites via trophallaxis and or grooming but could be transferred to cannibalistic individuals. However, it persisted in stained workers during and between molts. Termites survived and developed well during 110 days of oral administration of feed treated with 0.1% Nile Blue A solution, and formed supplemental reproductive. Strikingly, the dye was passed down from stained workers to supplemental reproductive, oviposited eggs, and even the hatched larvae were faintly colored. The effect of Nile Blue A on termite bait (Trelona, a.i. novaluron) was examined by measuring the survival rates of *R. flavipes* fed bait stained with the dye marker. There was no significant differences in mortality between groups of termites ingested stained-Trelona bait and control.

Stained termite through a 10-d ingestion of feed stained with 0.1% Nile Blue A solution retained visible blue color for at least 39 days following transfer to unstained feed.

Keywords: insect-marking; internal dye marker, Nile Blue A; non-repellent; bait

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1. Introduction

1.1. History of Marking Animals for Study

Marking animals has been an important technique for scientific studies involving organismal biology, ethology, ecology, and demography. Insect marking dates back to 1920 when researchers used paints, dyes, and stains in studies on insect population dynamics [1,2,3]. Insect marking has been used in mark-release-recapture or mark-capture studies to track insect movement in their natural habitats [4], to estimate insect population and territoriality [5], and to assess insect population dynamics [6], dispersal [7], trophic-level interactions [8,4], and other ecological interactions [9]. Insect marking has also been used in laboratory experiments for understanding interactions between individuals or groups within a larger population, particularly within social insect groups [10]. Due to the vast applications of insect marking, many marking techniques have been developed and evaluated.

Because of the extensive array of studies involving the marking of insects, a number of markers and marking techniques have been used to label insects. Insect markers range from conventional markers (tags, dust, etch, puncture, colored dye or glue, ink, paint) to isotope elements, immune-protein, and genetic markers [4]. Genetic markers are used to aid in experiments that target specific genes or genetic indicators, such as using green fluorescent protein variants to analyze the success of gene transfer or transgenesis [11]. Dust marking

involves exposing insects to dyed dust particles externally. Dust marking has been applied in mark-recapture studies aimed at measuring insect dispersal of mosquitoes and Hymenoptera [12]. Dust marking is easier and faster in applying, but can also be easily rubbed or blown off insect body. Dyes are also very useful markers which can greatly aid in the identification and labeling of insects. Dyes can mark insects externally by spraying on insect bodies or internally by feeding insects with victural stained with sufficient concentrations of the dye for visual detection. Dye makers are especially useful for visibly marking social insects for observation such as the primary focus of this paper, termites [13]. Inks and paints are similar in their ability to be useful for observing insects such as evaluating social wasps' behaviors [14].

Effective markers should be durable without inhibiting the insect's normal biology, nontoxic, inexpensive, easily applied, and clearly identifiable. The three common ways of marking insects are self-marking, individual-marking, and group-marking. Self-marking involves insects marking themselves by contacting or consuming stained victuals that are either natural substances, synthetic baits, or products. Individual-marking involves painting or labeling an individual insect through fine precision. Group-marking is the process of labeling or marking a group of insects, typically done by use of a spray or dust application. Insects can be marked internally or externally. Those conventional markers are generally applied directly to insect exoskeleton or epicuticle or indirectly acquired by insects, whereas the genetic markers and transposable elements need to be first tagged to the insects through consumption, contact, injection, or genetic-modification. Internal marking involves consumed dyes that are visible externally despite the dye product residing within the organism. Internal markings are beneficial in some studies as the mark cannot be brushed off or spread to another organism through simple contact. External markings involve a dye powder/solution or paint or other permanent marker to

be applied topically on the insect exoskeleton or epicuticle. The insect-marking techniques and the type of marker chosen for a study mainly depend on the nature of the experiment and the insect species.

1.2.Importance of Dye Markers for Marking Insects

Soluble dyes have been used very successfully as marking agents for insect studies. Dyes are inexpensive and reliable markers which can be easily added to a food source or bait allowing insects to self-mark. Many termite studies employ dyes to investigate various aspects of termite ecology and biology. Termites that feed on cellulose stained by a fat-stain become internally marked [3]. Internally marked termites have been used in mark-release-recapture and mark-capture studies of territoriality between nests, foraging distance, and population estimate. This technology has also been used in studies of nest-mate behaviors and toxin transmissions. Internal dyes are particularly useful for stain marking termites, which have miniscule size and relatively translucent body skin that make dye coloration very apparent. Once a dye is consumed by a termite, typically through a cellulosic medium, the dyed marking slowly becomes visible through the exoskeleton despite the dye residing internally within the termite gut tissues. This form of marking is not feasible for other insects such as coleopterans as their exoskeletons are generally much thicker and less transparent. Dyes which have been tested on termites include Sudan Red, Sudan Yellow, Sudan Black, Sudan Brown, Sudan Green, Napthol Green, Napthol Yellow, Napthol Black, Astrazon Red, Astrazon Green, Nile Blue A, Neutral Red, as well as others [15, 16, 17, 18, 19]. For example, Sudan Red 7B is an oil-soluble dye that is appropriate for short-term studies approximately of 15 days before dye-induced mortality may affect result [20]. Sudan Blue 35 is also considered a short-term dye for termites used in 5 to 6-day studies [21]. Short-term dyes provide notable dye visibility quickly yet result in significant mortality rates

faster than other long-term stains. Suitable stains should persist in the target organism visibly for up to a few months. Nile blue A and Neutral red have been used successfully for long-term assessments of more than 15 weeks [4].

1.3. Nile Blue A Selected Marker

Nile Blue A was chosen for the following bioassays due to its persistence and reliability as a stain [14, 18]. It is one of the most popular stains used for termites and has been tested on *R. flavipes* (Kollar) successfully in many studies [22, 23, 24, 25, 26]. Nile Blue A is an aqueous water and alcohol-soluble dye that stains and persist insect fat body and lipid in color of blue [3]. Previous study reported that termites stained with Nile Blue A at concentration of 0.25% caused no consequential mortality and the visual dye retention more than 50% of termites over a 4-month period [27]. Nile Blue A's properties, color, and long bodily retention make it a suitable stain for many termite studies. This study evaluates possible effects of Nile Blue A stain on the behavior and survival of *R. flavipes*. Five primary objectives were assessed. The first was to examine the effects of Nile Blue A on termite survival and change of body color. Second was to analyze the effect of Nile Blue A on the efficacy of Trelona active bait. Third was to determine if Nile Blue A can be transferred laterally from stained workers to naïve termites. Fourth, was to assess color retention of Nile Blue A stained termites as well as to determine if Nile Blue A can be transferred vertically amongst overlapping colony generations (i.e., vertical transfer).

2. Materials and Methods

2.1. Termite collections

Eastern subterranean termite, *R. flavipes*, were collected from field colonies in Auburn, Lee County, AL (GPS=32.616055,-85.4754492) using open-bottom underground traps described by

Hu and Appel (2004). The traps consisted of open-bottom plastic containers (18-cm high, 14-cm top diameter, and 11-cm bottom diameter) provisioned with corrugated cardboard rolls (15-cm high and 11-cm in diameter) which were set in the ground. The top of the container was closed with a plastic lid and covered with soil and leaf litter. Termites were extracted by gentle tapping of the cardboard rolls into a 10-gallon glass aquarium containing southern yellow pine blocks that had been soaked in water for 24 hours prior to termite introduction. Upon termite introduction, moistened brown paper towels were applied as covering over the wood block harborage, and the aquarium was then sealed and placed in a darkroom at 28-30ºC. At the time of each bioassay began, termites were gently tapped out of wood blocks into a large Petri dish where they were then counted and transported via a soft paintbrush into the appropriate bioassay dish as described below.

2.2. Visible marker and bait

Nile Blue A (≥75% wt/wt) certified by the Biological Stain Commission (Sigma-Aldrich), a visible internal marker, was used to prepare a serial dilution in distilled water to obtain 0.1%, 0.2%, and 0.3% (w/v) solutions. Microcrystalline cellulose blank tablets (non-active bait, NAB) and Trelona Control Termite Bait [TCTB] containing 0.5% Novaluron active ingredient were provided by BASF Corporation (Florham Park, NJ). NAB and TCTB were finely grounded up with a mortar and pestle into a fine powder, respectively.

Bioassays were performed under laboratory conditions of 28-30ºC, 70-80% RH.

2.3. Bioassay 1: Termite Nile Blue A survival and change of body color

Groups of termites (10 workers and 1 soldier) were introduced into each experimental unit (Petri dishes (5-cm in diam., 2-cm in height) provisioned with 1.0 g of BAB powder moistened with 55 drops (2.75 ml) of Nile Blue A solution of either 0.1%, 0.2%, or 0.3% concentration. NAB powder moistened with 2.75 ml of distilled water was used as control. Each treatment and the control had 5 replicates.. Experimental units were sealed with Parafilm to maintain moisture. Termite mortality was recorded weekly for 5 weeks. "Mortality" was defined as an individual that showed no movement of any body part. Body color observations were recorded with a Cannon Rebel T6 and Zarbeco ZC105 Megapixel Camera. Individual termite abdomen color was denoted using Pantone Matching System (PMS) color codes, with the selected individual color characterizing the general color observed over the termite abdomen. Mortality data were arcsine transformed before being analyzed using repeated measures ANOVA at α level of 0.05 to test statistical significance of weekly mortality among treatments..

2.4. Bioassay 2: Nile Blue A effect on Trelona efficacy

Groups of termites (10 workers and 1 soldier) were introduced into each experimental unit (Petri dishes: 5-cm in diam., 2-cm in height) provisioned with 1.0 g of TCTB powder moistened with 55 drops (2.75 ml) of either 0.1% Nile Blue A solution or distilled water and control. The treatment and control each had 5 replicates. Experimental units were sealed with Parafilm™ to maintain moisture. Termite mortality was recorded weekly for 5 weeks as described for bioassay 1. A two-sample t-test was done assuming equal variance to analyze if the group means from each treatment differed from one another.

2.5. Bioassay 3: Stained workers transmission of Nile Blue A to naïve workers

Termite workers were allowed to feed on Nile Blue A 0.1% stained NAB for 1 week in a Petri dish (9-cm in diam., 2-cm in height).Fourteen stained termites were transferred to another Petri dish (9-cm in diam., 2-cm in height) containing 28 unstained naïve worker termites**,** Whatman™ #1 filter paper cut to fit the bottom of the dish and unstained NAB both moistened with distilled water. Weekly for 4 weeks, the number of marked individuals was counted to confirm the transfer effect of this dye marker.cc.

2.6. Bioassay 4: Retention of color in workers and vertical transfer of Nile Blue A from stained workers to resulted supplemental reproductive and their offspring

Termites were collected from in-ground traps, brought back to the laboratory, extracted by gently tapping termite-containing cardboard roll into two plastic containers (16-cm in diameter, 10-cm high) and tested the same day of collection. Container A contained brown paper towel (Uline Kraft, Pleasant Prairie, WI) moistened with 0.1% Nile Blue A solution, container B contained brown paper towel moistened with distilled water as control. Each container had approximately 3,000 termites consisting of workers, soldiers, and nymphs.

Body color of termites in container A and B (control) was observed and color images were taken on day 10, 21, 55, and 110. Additionally, on day 110, termites in each containers were observed for caste development.

To determine Nile Blue A color retention, a group of 200 (approximately) stained termites after 10-d feeding in container A was relocated to a new Petri dish, which had 400 naïve worker termite and was provisioned ad libitum with undyed moist brown paper towel. The retention time of the dye marker was checked and imaged on day 3, 23 and 39.

3. Results

3.1. Bioassay 1 Termite Nile Blue A survival and change of body color

Termites exposed to Trelona non-active and active treatments showed no aversion to the baits. They naturally explored and consumed the compressed microcrystalline cellulose bait matrix. This observation is supported by a supplementary study run with this experiment which showed naïve termites introduced to a petri dish containing non-active bait, Trelona active bait, and Thousand Yellow Pine all under equal saturated conditions, did not avoid the baits, yet rather congregated on and around the active and non-active baits preferentially compared to the natural wood, Thousand Yellow Pine. During the first week of exposure, termites gradually became blue in color as the dyed bait became metabolized. Termites of the 3 treatments appeared to show the same approximate color after the first week of staining, except for termites of 0.3% treatment had a little more of the color within their intestinal track. Following the first week, the Nile Blue A concentration appeared to influence the relative blue coloration of the termites per week. Termites exposed to 0.3% treatment resulted in darker blue termites in week 2, 3 and 4. The termite of 0.2% treatment at week 4 demonstrated the same approximate color as the termite of 0.3% treatment at week 3. The termites of 0.1% treatment showed the slowest per week color increase compared to the 0.2% and 0.3% termites, yet at week 4 their color was almost equivalent to the termite of 0.2% treatment. Table 1 displays the color code values exemplifying the weekly color change. For termite survival, the weekly survivals varied slightly among treatments. Weekly ANOVA tests were conducted to compare Nile Blue A concentration mortality values to one another. Week 1 displayed no significant difference in mortality amongst Nile Blue A concentrations ($F_{3/16} = 1.651$, p-value = 0.217, $\alpha = 0.05$). Week 2 did display a significant difference in mortality amongst Nile Blue A concentrations ($F_{3/16} = 3.556$, p-value = 0.0382, $\alpha = 0.05$), however a Tukey test displayed no difference amongst concentration groups

except for between control and 0.3% Nile Blue (p-adj = 0.031). Week 3 also displayed a significant difference in mortality amongst Nile Blue A concentrations ($F_{3/16}$ = 7.538, p-value = 0.0023, α = 0.05), a Tukey test detected the significant difference exists between control and 0.3% Nile Blue (p-adj = 0.021) as well as between 0.1% and 0.3% Nile Blue (p-adj = 0.02). Week 4 showed a significant difference in mortality amongst Nile Blue A concentrations ($F_{3/16}$ = 17.59, p-value < 0.001, α = 0.05), and a Tukey test displayed difference amongst every concentration against the 0.3% Nile Blue A with p-adj values < 0.001.. Week 5 displayed a significant difference in mortality amongst Nile Blue A concentrations ($F_{3/16}$ = 13.56, p-value < 0.001, α = 0.05), and Tukey test displayed difference amongst every concentration against the 0.3% Nile Blue A: control vs 0.3% Nile Blue (p-adj < 0.001), 0.1% vs 0.3% Nile Blue (p-adj < 0.001), 0.2% vs 0.3% Nile Blue (p-adj = 0.002). The data show that from week 2 until week 5,concentration of 0.3% Nile Blue A caused significant greater mortality respectively to the other concentrations. The weekly ANOVA tests showed that the control, 0% Nile Blue A, never statistically differed in terms of mortality from 0.1% Nile Blue A or 0.2% Nile Blue A at any week in the study.

Table 1. Body color codes associated with termites continuingly feeding on Nile Blue A stained microcrystalline cellulose bait

Nile Blue A Conc.	Week 1	Week 2	Week 3	Week 4
0.1%	PMS 625 C	PMS 624 C	PMS 2149 C	PMS 2161 C
0.2%	PMS 624 C	PMS 2150 C	PMS 2180 C	PMS 2150 C
0.3%	PMS 624 C	PMS 2150 C	PMS 2161 C	PMS 534 C

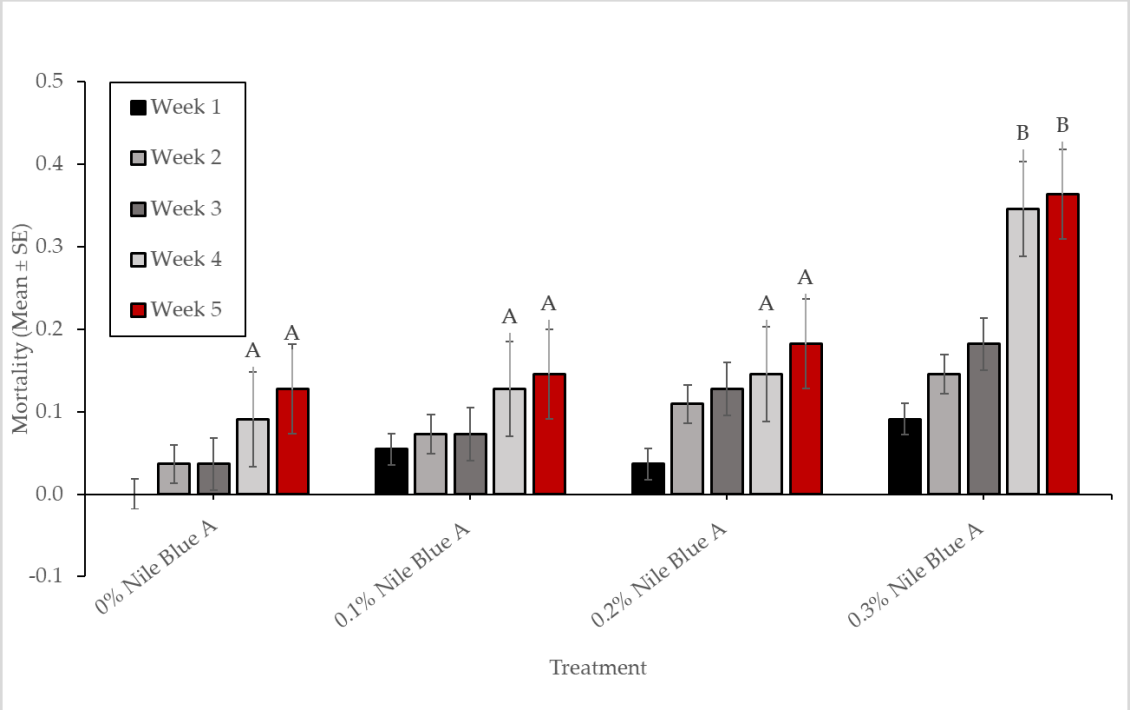

Figure 1: Mortality rates of continuously feeding termites according to concentration of the Nile Blue A over 5 weeks.

3.2. Bioassay 2. Nile Blue A effect on Trelona efficacy

The mortality of termites after feeding Trelona active bait stained with 0.1% Nile Blue A solution was investigated in comparison with that fed on Trelona active bait without Nile Blue A. For termites fed for 5 weeks, there was no significant difference in mortality (Figure 2) between the two groups ($T = -0.7845$, p-value = 0.4626, $\alpha = 0.05$).

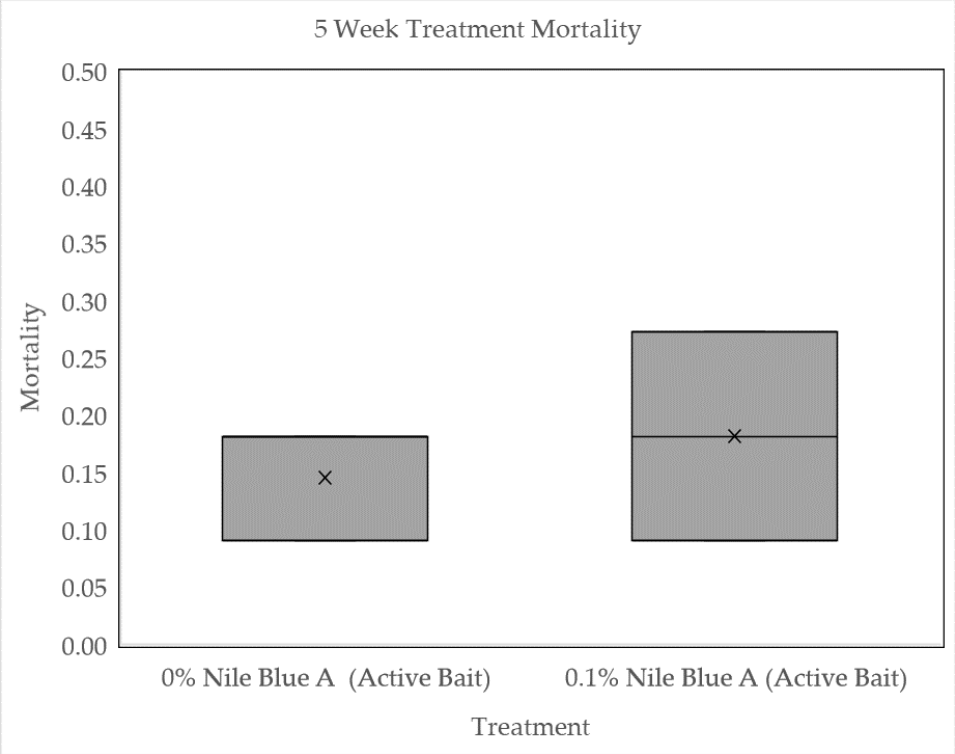

Figure 2. Mortality of 5-week feeding termites displayed via boxplot: non-stained Trelona bait vs. 0.1% Nile Blue A solution stained Trelona bait

3.3. Bioassay 3. Transfer of Nile Blue A from stained workers naïve workers

To determine whether transfer of Nile Blue A occurred between stained and unstained individuals, termites were fed food stained with 0.1% Nile Blue A solution for one week prior to being transferred to a Petri dish provisioned with unstained moist food and naïve termites. The ratio of the number of stained and unstained termites was 1:2. Upon introduction of stained termites, no unusual behavior or apprehension was observed in preexisting naïve termites towards stained workers. There was no change in color of naïve termites in the first two weeks of the trial. During the third week, coloration of the gut was observed in 2 out of the 28 unstained worker termites. Upon further investigation, it appeared one of the original 14 stained workers

had died, and its body was missing indicating cannibalism and consumption of dye/strain. There was no increase in staining at the fourth week or thereafter. Thus it was found that individuals stained with Nile Blue A did not transfer the dye marker by trophallaxis to other individuals in the same groups.

3.4. Bioassay 4. Retention period of Nile Blue A in stained workers and possible transfer of Nile Blue A from stained workers to subquently formed supplemental reproductives and even their offspring

Termites were fed with feed stained with 0.1% Nile Blue A solution, under no-choice conditions. Figure 3 shows the change in body color of termites fed for 10, 21, 55, and 110 days in container A, in comparison with the color of unstained termites in container B. As the feeding time increased, the color of termite body became increasingly bluer. As shown in the Figure 3, the termite's head also turned blue, indicating that Nile Blue A not only stay in termite's gut, but also spreads throughout the body where exist fat body or lipids. Supplemental reproductives, eggs, and hatched larvae were observed in both container A and B. Additionally, staining was noted in supplemental reproductives, eggs, and larval termites in container A. This result indicates that Nile Blue A stain not only stayed inside termite body when termite mold, but also carried on when the stained worker transformed into reproductive. Most interestingly, the stained supplemental reproductive passed down the dye marker to its offspring (eggs and larvae hatched from the stained eggs), which are visible in light blue color.

To determine the persistence of Nile Blue A in stained termites after 10-d feeding with feed stained with 0.1% Nile Blue A solution, the stained termites were mixed with naïve termites in ratio of 1:2. As they began to feed on unstained feed, the body color of stained individuals

became gradually lighter but remained remarkably visible for at least 39 days as shown in Figure 4.

4. Discussion

This study addressed four key objectives: to examine how the concentrations of Nile Blue A influence body color change and survival of termites stained internally by feeding, to understand whether Nile Blue A would affect the efficacy of Trelona active bait, to determine if Nile Blue A can be transferred from stained workers to naïve termites, to determine the color retention capabilities of Nile Blue A stained termites and whether Nile Blue A can be transferred to the next generation vertically. This study contributes towards the understanding of the influences of Nile Blue A on *R. flavipes* and provides insight into how Nile Blue A may be incorporated in future studies aimed at exploring Termite-Bait interactions.

4.1. R. flavipes Mortality and Color Change due to Nile Blue A

Previous studies involving termite staining have utilized Nile Blue A and have noted the capabilities of using Nile Blue A as a dye suitable for staining termites [15,19,22,27]. Bioassay 1 as illustrated above aided in our understanding on how oral administrtion of Nile Blue A changes color of termites at three key concentrations of 0.1%, 0.2%, and 0.3% Nile Blue A and how the concentrations affect termite survival. We observed a difference in staining rate during certain weeks and not during others. For all the three tested concentraitions, the first week of termite staining resulted in similar visible color and stain intensity for all termite groups. It was only as the staining time progressed that the apparent staining rate differed according to Nile Ble A concentrations. Starting from week 2, staining rate appears to be dependent on concentration.

The higher the Nile Blue A concentration, the faster the color staining will occur within a termite, as well as the darker the stain. This occurs until approximateley the fourth week when there is a leveling off in staining capability and how blue a termite can become. This may be due to the termites' gut tissues absorbing a maximum amount dye. Our data shows variability among termite stain group weekly mortality, however there was never a statistically different mortality mean amongst 0%, 0.1%, and 0.2% Nile Blue A concentrations at any of the 5 weeks. The significant difference displayed by the 0.3% treatment can be explained, as a previous study has shown 0.5% Nile Blue A can cause total mortality in termites in only 10 days [18], thus a staining percentage of 0.3% showing the mortality rate close to 40% in the 5^{th} week is consistent. A previous study reported 3-d ingestion of 0.1% Nile blue A stained food caused significant greater mortaltiy of R. flavipes at 18-27 post-treatment days [28]. However, the reported mortality was only more or less 2% greater in treatment (96%, 97% and 96%) than control (98%, 98% and 95%). In addition to the assessment of color change, the associated mortality rates are also worth noting. This information may support future studies which use Nile Blue A at concentrations around and below 0.2% for staining termites with the purpose of a long term, multi-week study.

4.2 Nile Blue A effect Trelona Efficacy

The second objective focused on identifying if Nile Blue A would affect the efficacy of Trelona active-ingredient, novaluron, bait product. The reason this question was posited is due to the nature by which the novaluron active bait is intended to interact with termites. The Trelona active bait does not kill termites immediately as it is designed to be non-repellent in order to promote the spreading of the chemical by termites through social interactions. Bioassay 1 showed Nile Blue A having no effect on termite mortality and blank bait. In bioassay 2, Nile

Blue A in addition to the Trelona active bait did not show significant mortality changes compared to termites exposed to just the Trelona active bait. This is significant because if Nile Blue A had increased early mortality in termites, the efficacy of the bait could be questioned as the intended social interactions necessary to spread the active ingredient may not have occurred. The results of bioassay 2 indicate Nile Blue A may be able to be applied to a non-reppellent bait for the purpose of monitoring termite-bait interactions of behaviors.

4.3. Color Transfer Relations

Color transfer between stained workers and naïve workers was the focus of the third objective. Would stained termites transfer blue coloration to naïve termites in the presence of no Nile Blue A? This question is pertinent in regard to the viability of Nile Blue A in future studies involving the behavior or monitoring of stained baits-termite interactions. Bioassay 3 exposed workers to stained bait until termites could be suitably stained, one week, then transferred to a container containing unstained bait and naïve termites. The results indicated stained termites did not transfer noticeable color to naïve workers by trophallaxis or grooming. This could be because the stained and unstained termites were in the presence of a current viable food source and may not have needed to share food between each other. A few newly semi-stained workers were noted during week 3, but close examination indicates that the stained naïve termites were cannibalistic individuals who hadeaten the stained workers. Cannibalism is a normal behavior for termites [29]. Our finding that Nile Blue A, as well as other fat-soluble dyes, cannot be trophallaxisically transferred from stained to unstained, but can be transferred to cannibalistic individuals agree with other studies [11, 29, 30]. This information may be useful in future studies which wish to know if a termite has consumed a stained product based solely upon the coloration of a termite. Studies examining termite-bait interactions may be able to observe termites and

recognize instantly whether or not a termite has either directly consumed a stained bait product or consumed the baits product through a termite who had.

4.4. Color Retention and Vertical Transfer

Bioassay four assessed color retention and transfer of color vertically. The results of bioassay four showed that termites who had previously 10-d ingestion of on stained food matter could y retain the color for at least 39 days after being moved to a location void of stained food. Although there was some concerns over long-term effect of the dye on insect development [31], our test indicates that the 110-d ingestion of food stained with 0.1% Nile Blue A solution had no harm effect on termites behavior and development. Stained workers not only devoloped into supplmental neotenic reproductive but also passed the color down to supplemental reproductive and subsiquently the eggs and larvae. Vertical transfer of Nile Blue A from female to eggs is also reported in spider, *Pholcus phalangioides* [29]. This result is notable because while workers have the ability to feed themselves, termite reproductives require workers to feed them. Thus if a worker has fed on stained food matter, the reproductive may also be exposed to the stain. Most notible, however, is the observation that the stain is not only able to persist visibly in the reproductive, but also in the subsequent eggs and hatched larvae; slight post reproductive staining was also observed. This information is valuable in showing the retention and spreading capabilities of Nile Blue A within termites post feeding. Future studies may explore the maximum capabilities of staining termites or residual staining.

5. Conclusions

5.1. Closing Remarks

Termites like many insects can be marked or stained for experimental use. Unlike many other insects, termites also possess the attribute that they can be internally stained yet visible externally due to their semi-translucent bodies. In this study, we explored one such internal stain, Nile Blue A. We found Nile Blue A to be a suitable stain for subterranean termites for the purpose of behavioral observation and termite-bait studies. We found Nile Blue A to stain termites in a concentration dependent fashion, while also not increase mortality rate in concentrations at and below 0.2% Nile Blue A compared with an untreated control. We found Nile Blue A in a concentration of 0.1% does not affect the efficacy of Trelona baits with 0.05% novaluron. Additionally, we noted no color transfer potential from stained workers to unstained workers via trophllaxis or grooming, but possibly if unstained termites ate the marked individual. Furthermore, we noted that Nile Blue A can be shared vertically from reproductive to eggs and larvae. These results provide insight into the usefulness of Nile Blue A as stain for termite studies, as well as a potential stain suitable for examining termite-bait interaction studies.

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CHAPTER 3

Video Analysis of Termite Colony, *Reticulitermes flavipes*, Throughout Exposure to Trelona Termiticide Bait

Abstract:

The Subterranean termite, *Reticulitermes flavipes* (Kollar), is an economic pest controlled through a variety of methods. One control method deployed against *R. flavipes* is termiticide baiting systems. These systems contain a bait which is comprised of a cellulosic matrix incorporating an active ingredient chemical which is typically a chitin synthesis inhibitor. While baiting systems have been shown to be highly effective at eliminating termite colonies, the exact interactions between termites and baits have been difficult to illustrate as both the bait and subterranean termites reside underground, occluding visual observations. In this study we exposed *R. flavipes* colonies to Trelona ATBS termite bait containing the chitin synthesis inhibitor, novaluron, within a confined enclosure and observed on a colony scale key termite behaviors and social interactions. This ranged from bait introduction to colony collapse with a contemporary video technology. We also assessed the movement speed of termites traversing a designated tunnel pathway throughout the experiment duration. We found a significant ($F_{1,88}$ =7.481, $p < 0.0001$) decrease in termite tunnel movement speed as days post bait introduction as days increased. This study documents for the first time, through visual observations, the understanding of the behavioral and social components involved in termite-bait interactions necessary for bait efficacy.

Keywords: Bait; Chitin Synthesis Inhibitor; Behaviors; Efficacy; Video Technology

1. Introduction

Insects are multifaceted creatures each with unique locomotion mechanics and behavioral characteristics. Much of the knowledge researchers have collected about insects as well as other arthropods has come from visual observations. Before the advent of modern technology, observations of insects needed to be made manually and recorded. Other than a paper documentation there was no way to record the actual visual observation seen. Contemporary technology has allowed for advancements in recording and visualizing movements and behaviors. Technologies which have allowed for researchers to visually record insects [1], track insect movements [2,3] create 3-D representations of insects from scans [4], radio tag eusocial insects [5], audio record insect acoustic sounds [6], as well as other technologies which have aided in our understanding of insects. Video recording technology in particular has aided in many insect associated research studies [7,8,9,10,11] The ability to view an insect or arthropod for extended periods of time or through difficult lighting conditions can prove invaluable in achieving newfound observational data.

Termites like other insects have been studied using video technologies [12,13,14,15]. Unlike other insects, termites are particularly difficult to observe as most reside cryptically within structures or in the case of subterranean termites, underground. The cryptic nature of subterranean termites makes observing their activities extremely difficult and technologically challenging. While subterranean termite observations have been made under laboratory and field conditions, the exact mechanisms involved in controlling subterranean termites is still poorly understood. One management method which is popular in controlling subterranean termites is the practice of termite baiting. Contemporary termite baiting products have been around since the 1990s. They have been a staple tool in controlling termites alongside liquid termiticide treatments. This is because unlike

liquid treatments which provide nearly immediate results after termite exposure or appropriate uptake [17], termite baits work through the use of the selected active ingredient [AI] working slowly, not causing rapid or immediate death. This is because the AI within a termite bait product is intended to be consumed and shared by termites through their natural behavioral interactions amongst each other. This allows for individuals from a colony to be exposed to the AI without having to consume the bait product at the site of placement allowing for colony collapse.

While bait products have shown to be highly effective at controlling subterranean termite colony populations [17,18,19,20,21,22,23], the exact mechanisms behind bait efficacy has been incomplete as underground visual data of termite bait interactions within a colony has not been documented. Many studies have attempted to evaluate termite bait efficacy through the use of mark-release-recapture methods [19,20,24,25,26]. This is because mark-release-recapture methods allow for colony population estimates to be made over a select period. In doing so, a termite colony's population which has been exposed to a baiting system in a nearby area can be monitored and recorded. While mark-release-recapture methods may yield valuable information in terms of colony presence, mark-release-recapture methods do no evaluate or document the actual interactions taking place between individual termites, the bait, and the termite colony.

Thus, this study aims to increase our understanding of mechanisms involved in achieving termite colony collapse as a result of termiticide bait exposure and termite-bait interactions. In other words, we investigated the effects of Trelona ATBS termite bait on sequestered enclosed *Reticulitermes flavipes* colony with the aid of a contemporary video technology. Our goal was to record and document behavioral and social termite interactions which have been speculated and implied in previous studies regarding termite bait efficacy pertaining to *R. flavipes* colony population collapse.

2. Materials and Methods

2.1. Insect

Eastern subterranean termites, *R. flavipes*, were collected from field colonies in Auburn and Opelika, Lee County, AL, from May through June 2019, using open-bottom in-ground traps described by Hu and Appel [27]. The traps consisted of capped open-bottom plastic containers (18-cm high, 14-cm top diameter, and 11-cm bottom diameter) provisioned with corrugated cardboard rolls (15-cm high and 11-cm in diameter) which were set in the ground. The top of the container was at ground level and covered with soil and leaf litter. The collected cardboard rolls were brought back in plastic buckets to Urban Entomology Research Laboratory on Auburn University campus. An additional termite colony was collected in Gainesville Florida at the University of Florida's Natural Teaching Lab on January 2nd, 2020 from a wooden log.

2.2. Experiment Design

2.2.1. Experimental unit

Termites were dislodged by gentle tapping of the cardboard rolls into glass aquariums (40.64 x 20.32 x 26.67 cm^3). Each unit (i.e. glass aquarium) contained 9 moistened (submerged in DI water for 24hr before placement) (15.24 x 3.81 x 1.2 cm3) of south yellow pine at one side weighing a total of ~368 grams. The estimated number of termites in each glass aquarium was ~ 4,000 to 5,000 individuals for the Auburn colony and ~3,000 to 4,000 individuals for the Gainesville colony. Termites from the Auburn collections were placed in the glass aquarium on 06-17-2020 to feed on wood blocks in order to allow for production of a functional colony with

all forms of caste structure from supplementary reproductive to immatures and eggs. Soil and bait were introduced into the set-up on 08-26-2020. However, the Gainesville colony was transferred into a large viewing tank (40.64 x 20.32 x 26.67 cm3) on 01-08-2020. All colonies were held at ~23 °C. In total one colony from the Auburn collection and one colony collected from Gainesville Florida were used in this experiment.

2.2.2. Viewing Experimental Colonies 1 & 2

The Auburn and Gainesville termite colonies (40.64cm x 20.32cm x 26.67cm) containing only wood blocks as harborage and approximately 3,000 to 5,000 termites devoid of soil were given a Trelona ATBS active bait (0.5% Novaluron) tablet. The Trelona ATBS active bait tablet was placed against the bottom of the aquarium's glass on the opposite side of the termite colony wood harborage (used as the nest and initial food supply) (Figure 1). The tablet (32.5g) was moistened with 40.0 ml of 0.1% Nile Blue A solution then placed into the glass aquarium (hereafter referred to as Viewing Experimental Colony) at the opposite of termite nest. Viewing Experimental Colony 1 represents the Auburn colony while Viewing Experimental Colony 2 represents the Gainesville colony. Moistened sterilized soil was introduced to cover the tablet and bottom of the aquarium until the top of the wood was completely concealed by soil. The viewing experimental colonies were then immediately placed on a shelf in the dark room over a rectangular cut-out made in the shelf which completely exposes the bottom of the aquarium while still supporting the bottom sides of the aquarium.

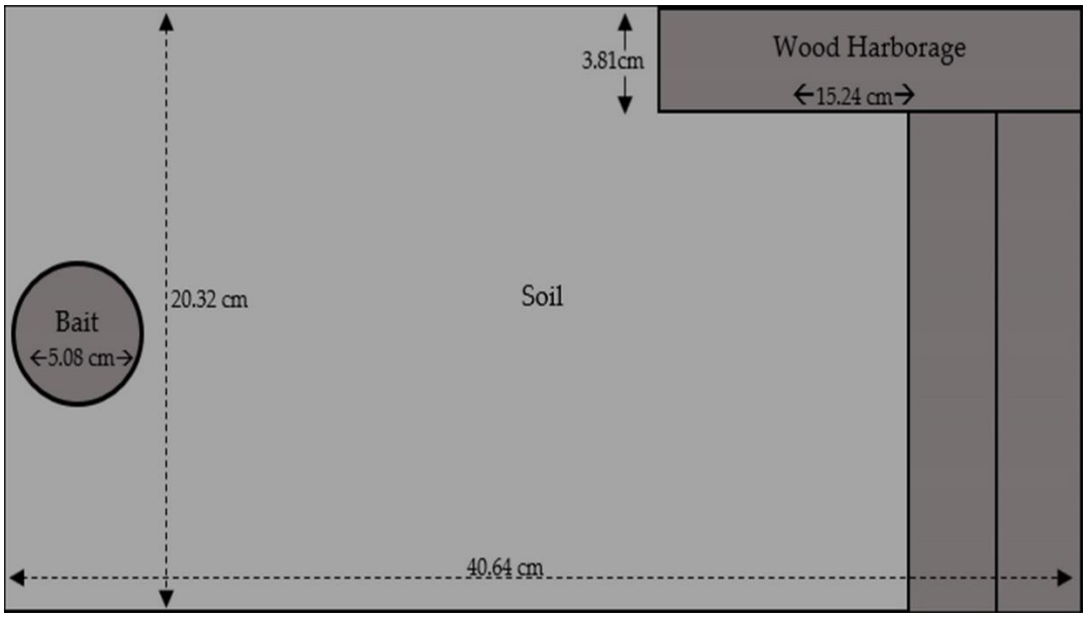

Figure 1: Visual diagram of the bottom of the Viewing Experimental Colonies

2.2.3. Video/Observation Equipment/Procedures

2.2.3.1. Video Equipment, Observations, & Recordings

Observations and recordings were made using one Amcrest NV4108E-HS 4K 8 Channel POE NVR device equipped with one WD Purple 6TB Surveillance Hard to store recordings and transferred to a computer for viewing and analysis. Two Amcrest UltraHD 4K (8-Megapixel) Varifocal PoE cameras hereafter referred to as “camera 1” and “camera 2” were used to film the bottom of the viewing colonies. In addition, one Tendelux A14 IR Illuminator 850NM IP65 CCTV light was used to provide infrared light to aid in camera image quality. One Cannon XA25 was used as behavior recording device.

2.2.3.2. Camera Positioning/Functions/Schedule

Camera 1 was set up and mounted directly underneath the viewing tank, and adjusted/focused to capture the entire bottom of the Viewing Experimental Colony container from the wood harborage at one end of the container to the Trelona bait tablet at the other end of the container. Camera 2 was also placed and mounted under the bottom of the container. However, it was focused and adjusted to capture the Trelona Bait tablet and its surrounding area exclusively. The Cannon XA25 was operated by hand and used to capture specific termite behaviors of interest.

Camera 1 and Camera 2 were used to film the bottom of the containers for the entire duration of the experiment (i.e. from soil and bait introduction until apparent colony collapse). The purpose of Camera 1 was to capture all the termites' activities on a larger colony scale and highlight key moments such as the inception of colony tunneling from the initial wood harborage to the Trelona bait tablet. Camera 2 provided a closer more detailed look into the interaction between the termites and the Trelona bait tablet, starting from the termites' first contact with the bait to the end of the experiment. The Cannon XA25 was used to capture individual and group behaviors. The Cannon XA25 provided a closer visual of specific termite behaviors and interactions at the control of the operator. The manual observations involved the physical manipulation of the Cannon camera to record the intended behaviors. Camera 1 and Camera 2 were set to record 24hrs a day throughout the duration of the experiment until colony collapse. The Cannon XA25 was used to capture behaviors during the first 2 weeks (14 days) of the experiment.

2.3. Termite Movement Assessment

In addition to the observational data collected from Camera 1 and Camera 2, termite movement speed (seconds) was assessed over time (days) for Viewing Experiment Colony 2. Termite movement speed data was assessed at 9 interval (day) periods throughout 21 days for Colony 2. At each day interval, 10 termites were timed traveling down a primary tunnel in between two tunnel openings 10cm apart in distance. The 10cm tunnel distance was calculated by measuring 10 worker termites' length from head to abdomen tip, averaging them, then that distance was multiplied by the number of termite body lengths from tunnel opening A to opening B (distance or measured tunnel) along the tunnel length. The termites selected and timed were all recorded moving along the same direction from the wood harborage towards the bait matrix within the tunnel. Termites selected for timing were required to continually move between the starting tunnel location and the finishing tunnel location without stopping. However, termites were permitted to bump or brush past other termites provided they were continually moving throughout the tunnel from starting location to stopping location. The speed data recorded was subjected to One-Way repeated analysis of variance (ANOVA) in RStudio (version 3.6.1) [28].

3. Results

3.1.2. Viewing Experimental Colony

Viewing Experimental Colony 1 experienced colony collapse within an approximate period of 9 weeks. Colony 1 remained under continued observation after apparent colony collapse and no increase in colony population or colony rebounding was observed through the remaining period of the experiment which was 3 months. Upon soil and bait introduction (i.e. Day 0), termites began to create tunnel networks until tunnels had spread all throughout the bottom of the aquarium and to the Trelona bait tablet (Figure 2). Termites then began to feed on the bait tablet

while also creating tunnel networks within the bait itself as evident upon bait tablet data collection where numerous tunnels and pathways were seen throughout the bait tablet. Proceeding bait discovery by the termites, the bait mass became a congregation location for the termites as large masses of termites were seen on and around the bait (Figure 2). Within the first 9 weeks of the experiment, termite tunnel locomotion activity gradually reduced until only the closest tunnels between the bait and the wood harborage were utilized. The other tunnels remained untraveled by termites. Thus, few termites were seen on or around the bait or wood harborage for the remainder of the experiment. Viewing Experimental Colony 1 was concluded 173 days with no surviving termites present. The bait was removed and weighed (21.44 g) corresponding to a ~35% reduction in mass. An unused bait tablet weighs ~33 g. The termite colony consumed ~40% of their initial wood harborage with 223.49 g of harborage left out of an initial weight of ~368 g (9 wood blocks normal weight = ~368g).

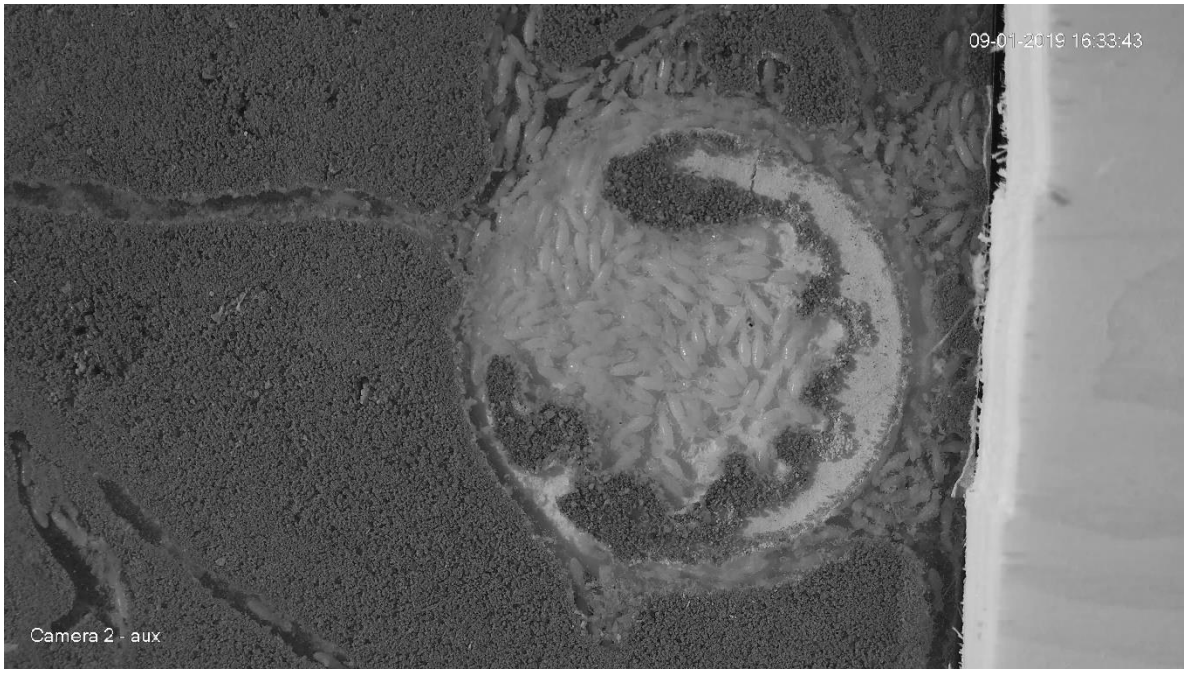

Figure 2: Termite congregation on and around bait of Colony 1 taken 9 days post soil and bait introduction. Termites can be seen underneath of the bait tablet as well as surrounding the perimeter of the tablet positioned closely with one another.

3.2.1. Viewing Experimental Colony 2

Viewing Experimental Colony 2 was concluded at colony collapse which was designated due to severe population decline at 47 days post soil and bait introduction. Colony 2 suffered similar fate as those observed under Colony 1. Nevertheless, numerous behavioral observations were recorded using the cannon hand camera. and are discussed below. Upon soil and bait introduction, termites began creating and forming tunnel networks as seen by Colony 1. The first termites' tunnel reached the bait 9 hours and 7 minutes after soil and bait introduction. The very first particle of bait taken from the tablet was immediately taken back to the wood harborage by the termite who broke the bait particle off with its mandibles. The cannon camera recorded termites consuming the bait, storing bait particles in tunnel walls, and transferring bait particles to the wood harborage. Other notable behaviors observed were naïve workers grooming stained workers (stained workers describe individuals which consumed bait matrix containing Nile Blue A, thus representing an infected worker), stained workers and naïve workers interacting co-habitably between one another, increased tunnel widths in primary tunnels leading to the bait and in between the wood harborage, nymphs near stained workers, group and individual cannibalism, stained workers feeding naïve worker via stomodeal, proctodeal trophallaxis, stained nymph interacting within the wood harborage, nymphs traveling between the bait and wood harborage, as well as naïve workers attempting to move an intoxicated stained worker. On the 47th day, the experiment was terminated, and careful excavation was done to recover survivors. There were 37 total surviving termites recovered. Of those 37 termites, 36 were workers and 1 was a soldier. No

nymphs were seen upon excavation however, a dead queen with mangled and damaged legs was found within the bait mass next to the sole surviving soldier. Termites recovered were slow and lethargic in movement as well as demonstrating bodily locomotion discontinuities. The recovered termites were stained blue or milky white in coloration. The bait was carefully extracted and weighed in at 30.84 g, and approximate reduction of 7% in bait mass. The termite colony consumed ~18% of their initial wood harborage with 302.41 g of harborage remaining out of an initial weight of ~368 grams.

3.2.2. Termite Tunnel Movement Speed Data

Termite movement speed was observed and analyzed for Viewing Experimental Colony 2. Figure 3 displays the mean speed in seconds of termite movement along the selected tunnel length. The graph shows decrease in termite speed as days increases. The movement speed data ceases at day 21 and does not continue due to ceased utilization of the primary tunnel proceeding day 21 termites stopped using the primary tunnel which was used for timing the termites. Termites instead used secondary tunnels on the outskirts of the container instead of the primary tunnels which were wider and provided a more direct path from the bait to the wood harborage. At day 40 no termites were recorded traversing tunnels instead only showing termites remaining in isolated pockets at tunnels entrances but not traveling throughout them. There was a significant difference ($F_{1,88} = 7.481$; $P = < 0.0001$) in speed times over days. An increase in speed time over days represents a decrease in mean termite tunnel movement speed as days post bait introduction increases.

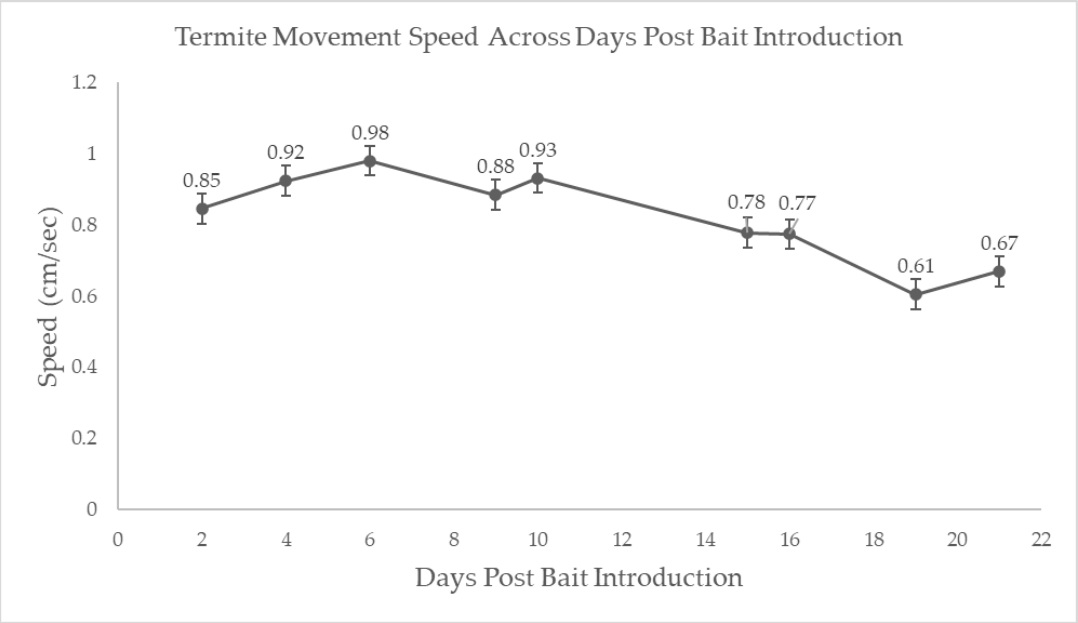

Figure 3: Termite tunnel movement speed over 9 individual day intervals. Graph displays mean of 10 termites per day interval traveling through a selected tunnel path. The graph shows a decrease in speed as the days since soil and bait introduction increases.

4. Discussion

This study aimed to increase our understanding of mechanisms involved in achieving termite colony collapse as a result of termiticide bait exposure and termite bait interactions through the use of video recording technology. This study found Trelona ATBS bait to be an effective bait for achieving colony collapse of subterranean termites *Reticulitermes flavipes*, while also documenting behavioral mechanisms and interactions between termites which support the established literature's stance on the importance of social interactions among termites in relation to bait efficacy[17,18,22,24,29,30,31,32,33]. This study documented healthy termite colonies population from bait and soil introduction to colony collapse. Many of the behaviors noted and

observed help to support the theories of how baits success utilizes the natural social interactions of termites leading to colony population suppression.

4.1. Termite Experimental Viewing Colony Overview

Upon soil and bait introduction into both Viewing Experimental Colony 1 and Viewing Experimental Colony 2 termites began to create tunnel networks in search of additional food sources apart from their respective wood harborages. Numerous tunnels were created branching out from the original wood harborages. The first termites reached the bait tablet in Trial Colony 2 after 9 hours and 7 minutes. Upon the discovery of the bait tablet, a worker termite immediately bit a piece of bait matrix and carried it all the way back to the wood harborage in its mandibles. The initial transportation of bait particle to the wood harborage is notable as this may be the worker termite alerting other termites at the nest of a potential viable food source. Additionally, this observation displays non-repellency from the initial worker towards the termiticide bait tablet, which is to be expected as the Trelona bait matrix is designed to be non-repellent towards termites. Within days after bait discovery, large masses of termites were seen feeding, tunneling and interacting on and around the bait. This occurred for both Viewing Experimental Colonies 1 & 2, with a visual representation being shown in Figure 2.

While not *Reticulitermes flavipes*, the subterranean termite species *Coptotermes formosanus* (Shiraki) have been known to radiate tunnel networks outwards until they reach a new cellulosic food resource then use that resource as a new center for outward expanse [34]. Given the aquarium being the scope of the available environment for the termites, outward expanse from the bait could not occur seeing as glass walls would be encountered. The termites did however remain congregated on and around the bait having found it to be a suitable food source. Our observations

showed no feeding aversion or termite repellency towards the bait throughout the whole duration of the experiment in both Trial Colony 1 and Trial Colony 2.

Video recordings showed termite placing bait particles into the tunnel walls of the most traveled tunnels. Tunneling termites remove soil particles from the front of a tunnel during excavation and deposit the particles further back either in a tunnel wall or at a deposition site. The storing of bait in the tunnel walls could have resulted due to the termites attempting to excavate and explore the bait matrix requiring a location to store the bait particles as they tunneled throughout the bait tablet. The bait particles however were stored through the whole length of the primary tunnels. The placement of bait particles in the tunnel walls could also be an attempt to store food throughout primary tunnels for later consumption. Notably though, the tunnel widths of the two most used tunnels (tunnels which allowed the shortest length between wood harborage and bait) were seen to be much larger than distal search tunnels. The Rhinotermitidae species *C. formosanus* have also been noted to invest more in primary tunnels than secondary tunnels given food size availability [35]. The primary tunnels between the bait and wood harborage were seen to have increased width compared to distal tunnels as the primary tunnels were observed to have enough width for up to 3 termites to be able to pass by each other compared to distal tunnels being only wide enough for one to two termites at a time. This may be the result of termites investing in the bait as an important food resource for feeding the nest colony members in the wood harborage.

Colony collapse was documented to have occurred in both Viewing Experimental Colonies 1 and 2. Colony 1 and Colony 2 yielded colony collapse at approximately 63 days and 47 days, respectively. This discrepancy can be explained by examining each colony. Viewing Experimental Colony 1 was incepted 67 days before soil and bait introduction, whereas Viewing Experimental Colony 2 was incepted only 27 days prior to soil and bait introduction. This could explain why

~40% of wood harborage was consumed for Colony 1 as compared to ~18% of wood harborage consumed by Colony 2. Colony 1 also had an estimated additional 1,000 termites compared to Colony 2. The difference in population may explain why Colony 1 showed a ~35% mass reduction in the bait tablet compared to the ~7% mass reduction recorded by Colony 2. Our experimental design allowed us to capture accurate points of colony collapse for termite colonies exposed to Trelona bait because we were able to observe the colonies as time progressed allowing for visual population indication of colony collapse to be seen. Not only did we document colony collapse, but we also found a deceased queen replaced from the initial wood harborage located within the bait matrix upon final colony dissection. The deceased queen appeared to be guarded by the only remaining soldier, however displayed damaged legs indicating potential over-grooming. The presence of a queen within the bait matrix may indicate the termites deemed the bait matrix a suitable location for the colony. This could be due to the cellulosic components and contents of the bait matrix which led to this potential relocation occurrence.

4.2. Termite Tunnel Movement Speed Data Overview

This study also examined termite individual speeds as they moved through a specific tunnel pathway. The purpose of this examination was to investigate the toxicity effects of the bait's active ingredient, novaluron, in a colony setting overtime. Novaluron is a chitin synthesis inhibitor designed to disrupt the production pathways of chitin as the target immature insects develops into adults. This disruption results in malformations of chitin which may affect the locomotion mechanics of the developing arthropod. In the context of this study, termite tunnel movement speed was seen to decrease as the days post bait introduction went by. This is to be expected, because as the days increase, so too does the spreading of the bait's active ingredient throughout the colony. Additionally, because the bait is designed to be slow acting, an increase in days allows

for toxicity within termites to increase. Thus, as the days continue to increase, the spreading of the bait and toxicity continues allowing for more toxic termites to be present as the days continue. Figure 3 displays the slowing of termite speed over the duration of the first 21 days. This is significant because following the first 21 days the primary tunnel which was used for tunnel speed data was no longer used by the colony member despite members continuing to travel between the wood harborage and bait along secondary smaller tunnels. *C. formosanus* subterranean termites have been documented to seal of close off or avoid certain tunnel areas in order to protect themselves, believed to be due to insecticide repellency or chemical factors associated with deceased termites [36]. Within our study however, bait repellency does not appear to be the reason for lack of primary tunnel traveling as workers were still seen traveling to and from the bait and wood harborage. The lack of primary tunnel traveling could have resulted from increased dead or toxic individuals or decreases in overall colony population. Even though there is no speed data for days continuing from day 21 to day 47, the information gained is still valuable. We see even before a tunnel is "closed" (no longer used by the colony members despite no obstructions) termites actively continue to use primary tunnels even while toxicity is occurring as evident by the statistically significant difference in speed mean values as days increase. Thus, despite toxicity onset, colony members still continue their tunnel routes and movements until the tunnel pathway is "closed", but by then increasing toxic individuals have already been traversing between the tunnel pathways and colony population has decreased showing few termites throughout the tunnel networks.

Key behaviors observed as mention in the results section are emphasized in contextualizing the importance of the role termite social and behavioral interactions contribute towards termite bait efficacy. Our study visually recorded natural behaviors believed to be responsible for the

success of bait transmission included naïve workers grooming stained workers, stained workers and naïve workers interacting naturally between on another, nymphs near stained workers, group and individual cannibalism, stained workers feeding naïve worker via stomodeal, proctodeal trophallaxis, stained nymph interacting in wood harborage, as well as naïve workers attempting to move an intoxicated stained worker. The utilization of Nile Blue A allowed us to see termites which had consumed the bait matrix. Hence stained workers represented those who contained not only Nile Blue A, but also the Trelona bait matrix's active ingredient chitin synthesis inhibitor, novaluron. Thus, stained workers interacting naturally with naïve colony members illustrates a non-discrimination amongst naïve and bait infected colony members in turn perpetuating the continued spread of the bait's AI through social behaviors amongst colony members. While termiticide bait studies have noted the importance of the social behaviors in regard to bait efficacy, our study has recoded and documented these natural behaviors on a colony scale which can be associated with success in bait efficacy for controlling subterranean termites.

5. Conclusions

5.1. Closing Remarks

This study aimed to increase our understanding of mechanisms involved in achieving termite colony collapse as a result of termiticide bait exposure and termite-bait interactions. We investigated the effects of Trelona ATBS termite bait on sequestered enclosed termite colonies with the aid of contemporary video technology. Our goal was to record and document the natural behavioral and social termite interactions which have been understood to occur yet hard to visually document given the underground nature of baiting systems and subterranean termite colony residing locations. We have successfully documented these behaviors which are important to our

understanding of termite-bait interactions as well as behaviors important for bait success and efficacy at causing colony collapse. We also documented a decrease in termite speed as days post bait introduction increased. This has not been done before and aids in reflecting the increase in colony toxicity as days go by through colony worker individuals tunnel travel speeds. Finally, we showed colony collapse with minimal surviving intoxicated caste members as well as the presence of a deceased queen replaced from the initial wood harborage and found within the bait matrix. These results provide insight and increased knowledge into the behavioral and social components involved in termite-bait interaction studies.

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