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MANAGING DEATH IN TERMITES

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MANAGING DEATH IN TERMITES

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
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2023

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ABSTRACT OF DISSERTATION

MANAGING DEATH IN TERMITES

Death of individuals from the same species represents potential risks from disease, predation or competition for animals. Diverse responses associated with death have evolved ranging from simply avoiding or being attracted to the corpses in solitary animals to complicated undertaking behavioral repertoire in eusocial insects. A systematic review in chapter 1 suggested cannibalism is an ancestral and widespread death-related behavior in all non-human animals. Termites are suggested to switch their undertaking behavioral responses from cannibalism to burial based on interactions between chemicals associated with death to balance risks and benefits associated with decomposition. In eusocial animals like termites with caste differences, conspecific death from different castes or different causes might deliver different types of risks to living members. However, whether termites are capable of distinguishing differences in conspecific death and responding accordingly remains unknown. In chapters 2 and 3, the eastern subterranean termite, *Reticulitermes flavipes*, was found to manage corpses from different castes or causes differentially based on both qualitative and quantitative differences in their chemical profiles. *R. flavipes* workers cannot distinguish dead individuals caused by termiticides apart from those killed by naturally occurring abiotic factors. Death by biotic factors accelerated the release of late death cues and the deployment of burial behavior in nestmate workers. Corpses of all castes were carried inside the nest and cannibalized when the postmortem time was <64 h. Burial behavior was consistently observed in highly decayed corpses regardless of castes. Additional behaviors, such as walling-off and movement of the corpse before burial were observed for 50% of soldier corpses. Postmortem chemical profiles showed that the early death cues, 3-octanone and 3-octanol, in worker corpses were significantly higher than in soldier corpses, while they were undetectable in nymphal corpses. In addition, we confirmed the existence of 3-octanol and 3-octanone in the head, thorax, abdomen, and hemolymph. Higher concentrations of early death cues were detected in the head and thorax than in the abdomen, suggesting the possible location of synthesis. Ultra-low temperature did not affect the amount or concentration across body parts, suggesting the synthesis of early death cues occurs prior to death.

Our findings suggest termites equipped with delicate risk assessments on different types of conspecific death based on chemical signatures: cannibalism was the prior

strategy dealing with freshly killed corpses to recycle nutrients and remove potential risks from pathogens or pesticides, whereas burial would happen when risks from disease or competition/intrusion overcome benefits associated with nutrition recycle. This study provides insights into the understanding of mechanisms of chemical-based “death recognition” in non-human animals, providing potential opportunities for further evolutionary studies of death-related behaviors.

KEYWORDS: evolutionary thanatology, eusociality, cause of death, death cue, caste, *Reticulitermes flavipes*

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MANAGING DEATH IN TERMITES

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DEDICATION

*To my grandparents, Hongchen Shi and Shuhua Qi,
may you rest in peace.*

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CHAPTER 1. EVOLUTION OF DEATH-RELATED BEHAVIORS IN NON-HUMAN ANIMALS: A SYSTEMATIC REVIEW

1.1 Abstract

A variety of strategies are used by animals to detect and respond to the corpses of their conspecifics. However, how these responses evolve and what factors influence the evolution of death-related behaviors remain unknown. Here, a quantitative review-based analysis of the evolutionary history of death-related behaviors among all non-human animals was carried out to further understand their evolution and their relationship to sociality.

Our bibliometric study revealed a consistent rise in interest as well as a promising integration throughout the currently dispersed field of death-related research.

Cannibalism was suggested as an ancient and common response to death in the reconstruction of ancestral states of death-related behaviors. Through a correspondence analysis, we also tested the “social complexity hypothesis” in death-related behaviors and suggested that the evolution of sociality precedes the expansion of death-related behavioral repertoires in terms of both scale and diversity but might not always be supported in some eusocial invertebrates.

Evolution of death-related behaviors might be influenced by the specific evolutionary history of sociality within taxa and also factors other than sociality. By addressing the study's biases and limitations, we suggested the necessity of well-documentation of death-related behaviors in broader taxa, especially in eusocial vertebrate animals, and encourage further investigations of factors associated with such behaviors, in order to enhance the reconstruction of evolutionary histories and provide a better understanding of the factors influencing the evolution of such behaviors.

1.2 Introduction

Encountering dead conspecifics brings with it risks of pathogen exposure, predation, and competition (Bijleveld et al., 2015; Osmond MM and de Mazancourt, 2013; Hamilton and Zuk 1982), leading to a myriad of strategies used to detect and respond to conspecific corpses (Gonçalves and Biro, 2018; Sun et al., 2018). Analogous to the fight or flight response, animals are generally expected to either approach or avoid conspecific corpses (Cannon, 1939). Attraction to dead conspecifics is widely observed in animals, including arthropods, amphibians, reptiles, birds, and mammals (Albeny-Simoes et al., 2014; Sun and Zhou, 2013; Valdes and Laidre, 2019; Santos et al., 2015; Swift and Marzluff, 2015; McComb et al., 2006; Bearzi et al., 2017). Avoidance responses are also widespread in both vertebrates (Stroud et al., 2014; Swift and Marzluff, 2015; Reggente et al., 2018; Prounis and Shields, 2013) and invertebrates (Rollo et al., 1994; Abbott, 2006; Neoh et al., 2012; Yao et al., 2009). So far, previous observations were mostly unconnected with little attention to their evolutionary history (Anderson et al., 2018). The diversity of animals' responses to their dead conspecifics raises a question: how do these responses evolve?

To answer such a question, comparative studies across extant taxa are required. Previously, studies have shown an array of responses to dead conspecifics in social insects (Sun and Zhou, 2013; López-Riquelme and Fanjul-Moles, 2013), birds (Curio, 1993), elephants (Gonçalves and Biro, 2018), cetaceans (Bearzi et al., 2017) and non-human primates (Anderson et al., 2018), as well as species across a broad taxonomic range (Peil and Stewart, 2016; Reggente et al., 2018; Gonçalves and Biro, 2018).

Empirical studies across territorial isopods and social caterpillars suggest that avoidance behavior following fatty acid “necromones” can be traced back to the common ancestor of insects and crustaceans, approximately 420 Mya (Yao et al., 2009). On the basis of current findings across broad groups of animals, it remains unclear whether any traits are phylogenetically ancestral states of death-related behaviors.

Next, it is worth questioning what factors influence the evolution of death-related behaviors. Narrowly speaking, death-related behaviors are usually not considered rigorous communicative behaviors, yet they share similarities with animal communications, in which receivers collect and respond to a variety of cues released by the sender (in this case, dead conspecifics) (Hölldobler and Wilson, 1990). Such similarity makes it possible for hypotheses/explanations of the evolution of communication complexity to be applied to death-related behaviors. To detect and deal with death of other conspecifics, invertebrate animals rely on effective perception of death-related cues which could elicit stereotypical behavioral responses, whereas vertebrate animals employ a sensory-cognitive process in death recognition, and the evolution of such perception and cognition system in death recognition has been suggested to be driven by the social environments (Sun et al., 2018; Gonçalves and Biro, 2018), implying a potential correlation between social complexity and the complexity of behavioral responses (Bradbury and Vehrencamp, 1998; Gonçalves and Biro, 2018). The “social complexity hypothesis” is intuitive suggesting that more complex societies facilitate more complex communicative systems. In vertebrate animals, most of the evidence comes from studies of vocal communication as the primary indicator of communication complexity, for example in birds (Freeberg, 2006), bats (Wilkinson,

2003), rodents (Lima et al., 2018), whales (May-Collado et al., 2017), and primates (McComb and Semple, 2005). In invertebrate animals, the evolution of eusociality in Hymenoptera has been suggested to require genes associated with elaborated chemical signaling systems, for example, development of exocrine glands and signal transduction (Woodard et al., 2011).

On the contrary, complex communicative systems do not necessarily co-evolve with social complexity, but perhaps evolve for other reasons and in turn, lay a necessary foundation for social evolution, for example, habitat, predation, species recognition, “neutral” evolutionary processes (Freeberg et al., 2012; Ord and Garcia-Porta, 2012). For example, a habitat with background visual or acoustic noise may influence the delivery of visual or acoustic signals, animals might adapt to it by changing sound frequency or speed of movements, for instance, Urban great tits, *Parus major*, increase their minimum voice frequency at noisy locations (Slabbekoorn and Peet, 2003). Predation is another possibility that might shape the communicative systems, such as in Túngara frog, *Engystomops pustulosus*, during the mating season, males would seize mating calls and only make basic calls under higher predatory risk from bats (Ryan et al., 1982). Species recognition between congeneric species, especially with similar appearance and ecology, has been suggested to promote the complexity of communicative systems (Freeberg et al., 2012; Ord and Garcia-Porta, 2012). Allometry of physical organs is suggested to influence signal production, for example, larger animals with longer vocal tracts might generate different types of voices compared to small ones (Frey et al., 2011). The “social complexity hypothesis” remains unexamined in death-related behaviors. Does the complexity of death-related behaviors always increase gradually with the evolution of

sociality or vice versa? Previous reviews have shown that higher levels of sociality may be associated with more complex repertoires of death-related behaviors, for example, cetaceans and other aquatic mammals (Reggente et al., 2018) or eusocial and non-eusocial insects (Sun et al., 2018). These comparative studies only provide correlation between death-related behaviors and sociality in specific taxa groups but couldn't distinguish the "social complexity hypothesis" and the "precursor hypothesis". To test the social complexity hypothesis requires not only the link between social and behavioral complexity but also direction of causality, which in this case if the evolution of sociality could predate the emergence of diverse death-related behaviors (Freeberg et al., 2012).

Most recently, Evolutionary Thanatology has emerged as a new research front to expand the traditional focus of thanatology (the study of death and dying) beyond humans to include all animals and comparative approaches that may reveal broader impacts of death on animal behavior and evolution. One of the aims of this new field is to search the links between death-related behaviors in non-human animals to complex funeral practices in modern human beings from an evolutionary perspective, answering questions such as which death-related behavior would be ancestral, does death-related behavior evolve from chemical-based responses, then emotional, to cultural death-related rituals (Anderson et al., 2018). To achieve this goal, comparative studies across all non-human animals would be essential, seeking the evolutionary root of death-related behaviors and reconstructing their phylogenetic relationship. In this study, following PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines, we carried out a quantitative literature review focusing on behavioral responses to dead conspecifics in non-human animals to address the ensuing three

questions: 1) how diverse are animals' behavioral responses to their dead conspecifics, 2) how do these responses evolve, and 3) if there is any relationship between sociality and evolution of death-related behaviors, specifically, if social animals require more complex death-related behaviors than solitary animals? To achieve that, we 1) conducted a bibliometric analysis of conspecific death-related responses among all non-human animals, 2) reconstructed ancestral states of death-related behaviors and cues, and 3) examined if sociality is a factor preceding the changing of animals' behavioral responses to death.

1.3 Materials and methods

1.3.1 Data collection

A systematic review was performed following Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines (Moher et al., 2009). The PRISMA checklist is attached as supplementary material (Table S1) and a flowchart illustrating the article selection process is provided (Figure 1.1).

An article search was conducted in the Google Scholar database. The search included all articles present in the database as of Nov. 10, 2021. Articles from different scholarly sources were collected, including primary (original documents containing firsthand information about a topic, i.e. original research papers, case reports), secondary (summary of the existing state of knowledge in a field at the time of publication, i.e. reviews), and grey sources (unpublished papers, including dissertation and thesis (though these are also considered primary sources, we categorized them as grey literatures for the convenience of our data extraction and statistical analysis in this quantitative review.)). A series of keywords related to death recognition was used in article identification (Table

S3). In brief, keywords from the included list were searched one by one for exact matches from anywhere in the article. All articles identified through keywords were collected for screening. No language restriction was applied for literature searching, although the search was conducted in English. Sentences containing keywords were used for screening.

First, articles were automatically removed based on the use of excluded keywords, including articles in non-biological fields (for example, literary works such as poems), cell/organ level death, human death, and death not in conspecific individuals (Table S3). Then, duplicate articles were removed in Endnote X9 (Clarivate Analytics, PA, USA). Finally, articles that did not meet all the following criteria were manually excluded: 1) article involves a biological field, 2) article contents pertain to non-human animals, 3) article examines death on the organismal level (not cell- or colony-level death), 4) article examines conspecific death. All returned articles from screening were included in our quantitative study.

1.3.2 Summarizing research topics using co-citation analysis

A summary of published information was performed for all included articles, including publishing years and types of scholarly sources. Visualization was conducted in GraphPad Prism (version 9.1.2 for MacOS, GraphPad Software, San Diego, California USA).

A co-citation analysis would uncover important works crossing disparate fields and provide a forward-looking evaluation of document similarity (Trujillo and Long, 2018), therefore selected in this study to explore research topics related to death

recognition based on reference information. Articles from primary sources with access to cited references in the Web of Science Core Collection database (Clarivate Analytics, PA, USA) were used in this analysis (n = 191 out of 299) with a total of 6261 references retrieved. Of these references, 162 were cited by 5 or more of the other references included in the analysis; these references were selected for network visualization. A co-citation network of selected references from these articles was visualized and clustered in the software VOSviewer 1.6.17 (Leiden University, Leiden, Netherlands). Resolution parameter was kept as default (1.00).

1.3.3 Reconstructing ancestral states of death-related behaviors and cues

To investigate the evolution of death-related behavioral responses and cues, an ancestral state reconstruction (ASR) was conducted in Mesquite 3.70 (<http://www.mesquiteproject.org/>) with equally weighted unordered parsimony.

Research associated with death recognition of a specific species was defined as a case. Cases were extracted from abstracts, hypotheses, objectives, or content containing keywords in articles. Taxonomic information (species, genus, and family) of research objects was collected. Behavioral repertoires of responses to conspecific death and associated stimuli (death-related cues) were summarized and defined based on previous knowledge of the research field and refined by co-authors (Table 1.1). Based on the nature of stimuli, death-related cues were identified as either chemical, tactile, or visual. Based on the tendency of individual movement towards or away from dead conspecifics, behavioral responses were categorized as either attraction, avoidance, or no-direction. Responses were further separated within groups: within attraction, responses were

subcategorized as either burial, cannibalism, removal, sexual, grooming, aggression, carriage, aggregation, or attracted; within avoidance, as either avoiding, food aversion, or sheltering; and within no-direction, as either ignoring, vocalization, or shaking (Table 1.1). Behaviors were defined based exclusively on the end result of the act on a corpse. For example, to remove dead nestmates from the nest (necrophoresis), workers must “carry” the corpse, a common strategy used to deal with the dead nestmates in bees and ants (Wilson et al., 1958; McAfee et al., 2018). We regarded this action as removal rather than carriage (defined here as “carrying or holding dead conspecifics, but not for disposal”) to limit the dependency between them. For each case, behaviors were binarily scored as a ‘1’ if present and ‘0’ if absent (Table S4). Data were binarily summarized on the species, genus, and family levels. Binary data of species were used in ASR.

ASR was based on a phylogenetic tree of all retrieved species generated in Phylot (<https://phylot.biobyte.de/>) using lineage information from the NCBI taxonomy database (<https://www.ncbi.nlm.nih.gov/taxonomy>). Visualization and annotation of phylogenetic trees were performed in iTOL (version 6.4, Letunic and Bork, 2021). For illustration, the earliest common ancestors of taxonomic rank at or above the family level with the most parsimonious present state were labeled on a family-level phylogenetic tree.

1.3.4 Relationship between sociality and death-related behaviors

A correspondence analysis (CA) was performed to investigate the relationship between sociality and death-related behavioral responses. Sociality of each species was categorized as solitary, presocial, or eusocial (Table 1.1). In this study, solitary animals are the ones showing no association with conspecifics outside of courtship and mating.

Eusocial animals are defined by 1) reproductive division of labor, 2) overlapping generation, and 3) cooperative brood care (Wilson, 1971). Presocial animals are defined as those showing non-reproductive interactions with conspecifics that were not eusocial. Subsociality and parasociality are included in presocial animals but not separated in this study. The criteria used to distinguish them, parental care, and nest-sharing, are used to identify presocial species in some cases. Each sociality group was subcategorized as vertebrate and invertebrate animals.

Behavioral repertoires were pooled among all taxa within each sociality level and their size was determined based on binary counts. A one-way ANOVA was performed to compare average behavioral repertoire size across the three levels of sociality between vertebrate and invertebrate animals used in our analysis. Correspondence analysis was carried out and visualized in R 4.1.0 using the FactoMineR (Lê et al., 2008) and factoextra (Kassambara and Mundt, 2017) packages. To test the correlation between sociality and death-related behaviors, sociality was also reconstructed based on species data following the methods described in the previous section. Presocial and sociality were colored for the whole clade following the reconstructed taxa of common ancestors. Size of behavioral repertoire was defined as the number of behaviors present at each taxonomic rank.

1.4 Results

1.4.1 Summarizing research topics using co-citation analysis

Publishing information of all returned papers was summarized by scholarly source and publishing decade (Figure 1.2A&B). Based on the PRISMA protocol, a total of 299 articles containing 495 cases were included in this review, of which most were from

primary scholarly sources (articles: 228 out of 299; cases: 282 out of 495; Figure 1.2A). After the first article was identified in 1958, the number of published articles and cases increased every decade with the largest increase occurring during the 2010s (from 2010 to 2019) with 146 articles and 287 cases identified (Figure 1.2B). Although only two years of data were available for the 2020s, 47 articles and 74 cases were identified during this time span.

A co-citation network map was constructed based on the references used by primary papers selected to identify major topics in death recognition (Figure 1.3). Co-citations were measured as the frequency with which two references were cited together by subsequent articles. Each node represents a cited reference and links between two nodes indicate that the linked references have been cited together by the same subsequent reference. The size of a node indicates the frequency with which it has been cited. References were clustered together based on their comprehensive closeness and each reference could only be in a single cluster, resulting in five clusters labeled with different colors (Figure 1.3). “Total link strength” was defined as the total number of times a reference was cited by other references included in the analysis, regardless of clusters. After checking the titles and abstracts of the ten references in each cluster with the largest total link strength, the five clusters were separated into two main groups based on their research subjects. The first group contained three clusters with 114 references, including the red (62 references), green (39 references), and yellow (13 references) clusters, with 110 references for invertebrate animals. The second group contained two clusters with 48 references, including the blue (38 references) and purple clusters (10 references), with 32 references for vertebrate animals. The two references with the largest total link strength

in each cluster were labeled in Figure 1.3. In the red cluster, the top two references were Wilson et al., 1958 (total link strength = 546) and Howard and Tschinkel, 1976 (total link strength = 440); in the green cluster, Visscher, 1983 (total link strength = 552) and Wilson-Rich et al., 2009 (total link strength = 227); in the yellow cluster, Yao et al., 2009 (total link strength = 280) and Rollo et al., 1994 (total link strength = 190); in the blue cluster, Douglas-Hamilton et al., 2006 (total link strength = 201) and Biro et al., 2010 (total link strength = 189); and in the purple cluster, Pinel et al., 1981 (total link strength = 94) and Fox, 1975 (total link strength = 82).

1.4.2 Reconstructing ancestral states of death-related behaviors and cues

Death-related behaviors and cues were reconstructed in 273 animal species (104 families) to explore their diversity and evolution (Figure 1.4). The phylogenetic tree was rooted in Bilateria and possessed two main clades: Deuterostomia (54 families, mainly in Vertebrata with 53 families, including lampreys, sharks, amphibians, reptiles, birds and mammals) and Protostomia (50 families, mainly in Arthropoda with 49 families, including spiders, crustaceans and insects). Parsimony reconstructions suggested cannibalism in response to dead conspecifics as the ancestral state for Bilateria. In Deuterostomia, Vertebrata was suggested as an origin for avoidance behavior, Batrachia (frogs and salamanders) and Episquamata (lizards and snakes) as origins for sexual responses, and Catarrhini (old world monkeys) and Whippomorpha (cetaceans and hippos) as origins for carriage behavior.

In Protostomia, Crinocheta (territorial isopods) was suggested as an origin for avoidance behavior, Acridoidea (grasshoppers) as an origin for ignoring dead

conspecifics, Polyphaga (beetles) and Chalcidoidea (a group of parasitoid Hymenopterans) as origins for sexual responses, and Paguroidea (hermit crabs) and Collembola (Entomobryidae and Onychiuridae) as origins for attraction behaviors. Corpse burial was suggested to have originated in the termite family Termitidae. Apidae and Formicidae were suggested as origins for corpse removal behavior, while Vespidae was suggested as an origin of aggressive responses. Possible origins for grooming, food aversion, sheltering, vocalization, and shaking responses were not suggested in any taxonomic rank higher than family.

Regarding the cues used in death recognition, reconstructions suggested chemical cues as the ancestral state for Bilateria, while Neognathae (birds) as an origin for visual cues. Origins for tactile cues were not reconstructed in common ancestors of taxonomic rank higher than family. The presence of each behavioral response was also binarily summarized on the species level (Figure 1.5). Corpse removal (67 species), sexual responses (66 species), cannibalism (61 species) and avoidance (53 species) appeared most often across the 273 species examined. Among those exhibiting corpse removal, 60 out of 67 species were ants or bees. Sexual responses were recorded in 17 species in Arthropoda but 46 species in Chordata. Cannibalism was recorded in 36 species in Arthropoda and 24 species in Chordata. Finally, avoidance was recorded in 38 species in Arthropoda but only 15 species in Chordata.

1.4.3 Relationships between sociality and death-related behaviors

A comparison of behavioral responses across sociality statuses and a correspondence analysis were performed to investigate the relationship between sociality and behavioral

responses to conspecific death. The summary showed that eusocial invertebrates possessed significantly larger behavioral repertoires than presocial and solitary invertebrates on the species, genus, and family levels, while presocial vertebrates possessed larger behavioral repertoires than solitary vertebrates at the same classification levels (Figure 1.6, all-pair comparisons with Tukey-Kramer HSD, $p < 0.05$). The repertoires of presocial vertebrates consisted of approximately two behavior types (2.09 ± 0.14 on species level, 2.21 ± 0.18 on genus level, 2.55 ± 0.26 on family level), while solitary vertebrates consisted of approximately one behavior type (1.04 ± 0.19 on species level, 1.07 ± 0.27 on genus level, 1.33 ± 0.48 on family level). The behavioral repertoires of eusocial invertebrates consisted of approximately two behavior types per species (1.89 ± 0.13 within 88 species) and per genus (2.35 ± 0.20 within 51 genera), but more than five behavior types per family (5.13 ± 0.59 within 8 families) (Figure 1.6&Table 1.3).

The correspondence analysis (Figure 1.7) showed that eusocial invertebrates and presocial vertebrates were associated with more death-related behavior types than solitary animals from either clade. Solitary species were only strongly associated with one behavior type, sexual responses; presocial vertebrate species were associated with 6 behavior types- attraction, food aversion, aggregation, grooming, vocalization, and carriage; eusocial invertebrate species were associated with 4 behavior types- burial, removal, sheltering, and shaking. Cannibalism, avoidance, aggression, and ignoring were located closer to the center of the biplot than other behaviors, suggesting that these response types show no association with any sociality status, with the exception of ignoring, which showed a weak association with solitary invertebrates.

Presociality was reconstructed in the common ancestors of Eutheria, Neognathae, Obtectomera, Blattoidea and Eumalacostraca. Eusociality was reconstructed in Termitoidae and Aculeata. Suggested origins for sexual responses were all located outside presocial and eusocial clades. Suggested origins for burial and removal behaviors were located exclusively in the eusocial clades, while suggested origins for carriage were located exclusively in the presocial clades (Figure 1.4).

1.5 Discussion

1.5.1 Summarizing death-related topics: integration in a divided field

All studies associated with death have been integrated into this new inter-discipline field of Evolutionary thanatology aiming to combine all these disparate topics to improve our understanding of the influence of animals' death on their living conspecifics and provide a broader evolutionary perspective on it (Anderson et al., 2018). Questions from both biological and sociological perspectives are intended to be addressed in this field, ranging from the fundamental mechanisms of how non-human animals recognize and respond to their dead conspecifics to the emergence and evolution of the cognitive understanding of death in human beings. Our summary of articles published in the field of death-related behaviors revealed near-exponential growth in the number of published articles, which shows a large spike in interest in the field during the past decade (2010-2019) (Figure 1.2B).

The results of our co-citation analysis indicate that topics in the field of death-related behaviors are divided by research subject, specifically, between vertebrate and invertebrate animals (Figure 1.3). Clusters in our co-citation network were divided into two groups based on their relative positions to one another. The first group contained

three clusters (red, green, and yellow) and consisted of references related to invertebrate animals, while the second group contained two clusters (blue and purple) and consisted of references related to vertebrate animals. In the first group, the red cluster covered references related to chemical-based corpse management, primarily in ants and termites, and was the largest single cluster (Wilson et al., 1958; Howard and Tschinkel, 1976). The green cluster primarily included references focusing on functions and mechanisms of undertaking and hygienic behavior in bees (Visscher, 1983; Wilson-Rich et al., 2009; Trumbo et al., 1997). The yellow cluster contained references examining fatty acids as necromones and predation cues across a variety of invertebrate animals, such as Collembola (Yao et al., 2009; Nilsson, 2004), social caterpillars (Yao et al., 2009), cockroaches (Rollo et al., 1994 and 1995), ants (Akino and Yamaoka, 1996), bumblebees (Abbott, 2006), and hermit crabs (Rittschof et al., 1992). In the second group, the blue cluster focused on death recognition in vertebrate animals, including observations of behavioral responses, mechanisms of said behaviors, and the concept of death (Douglas-Hamilton et al., 2006; Biro et al., 2010; Anderson, 2011). The purple cluster was the smallest with only ten references associated with it and included studies examining cannibalism on dead conspecifics, primarily in rats.

Historically, the study of death-related behaviors has been divided between vertebrate and invertebrate groups, with each group examining its own set of topics. With increased interest in the field, however, recent effort has been placed into uniting these two halves. In the past decade, comparative studies and reviews examining animals' responses to conspecific death and their underlying mechanisms have been conducted within and between vertebrate and invertebrate animals (Sun and Zhou, 2013; López-

Riquelme and Fanjul-Moles, 2013, Anderson, 2016; Piel and Stewart, 2016; Gonçalves and Biro, 2018; Reggente et al., 2018). In the following decade, it is reasonable to expect further integration within the field alongside a continuous increase in research interests.

1.5.2 Ancestral state reconstruction suggests that cannibalism is a widely spread and ancient death-related behavior

Ancestral states of death-related behaviors and cues were reconstructed and showed diverse behaviors in response to dead conspecifics across the animal kingdom (Figure 1.4). Although avoidance of conspecific corpses can directly eliminate associated risks, some animals still choose to approach the dead to actively manage these risks and even gain benefits from them. One example of attraction to dead conspecifics to limit potential risks is the undertaking behavior observed in eusocial insects, in which dead nestmates are either buried or removed from the nest (Sun et al., 2018; López-Riquelme and Fanjul-Moles, 2013). Attraction to dead conspecifics or death-related cues as a means of information access has been suggested in a variety of animals, including the African savannah elephant *Loxodonta Africana*, which directly contacts the dead with its trunk tip potentially to acquire gustatory information (Merte et al., 2009), and American crows, *Corvus brachyrhynchos*, which approach conspecific corpses to learn of the presence of novel predators (Swift and Marzluff, 2015). Moreover, animals can be attracted to the dead for direct benefit, as in land hermit crabs, *Coenobita* sp., which are attracted to the odor of dead conspecifics to locate new shells (Small and Thacker, 1994). Even sexual responses to death (also known as Davian behaviors or intraspecific necrophilia), which are usually considered as “behavioral mistakes” influenced by still-active sex

pheromones (Costa et al., 2010), could also bring direct benefit to animals. In the Amazonian frog *Rhinella proboscidea*, males can compress the abdomens of dead females and fertilize the ejected oocytes to overcome potential losses during the breeding season (Izzo et al., 2012). Among all described responses, our results showed corpse removal, sexual responses, cannibalism, and avoidance as the four most common at the species level (over 60 out of 273 species) (Figure 1.5). The number of species cannibalizing their dead conspecifics was generally balanced between the Protostomia and Deuterostomia clades, compared with the bias observed in the other three. For example, corpse removal was recorded in 67 species, however, 60 of these species belong to the order Hymenoptera. Therefore, our results indicate that cannibalism is the most taxonomically widespread behavioral response to conspecific death.

Our reconstructions of death-related behaviors suggested cannibalism on dead conspecifics as an ancient response in Bilaterian animals (Figure 1.4). A cannibalistic strategy has been suggested as a normal phenomenon across taxa that is influenced by factors such as food availability, population density, the statuses of “victims” and cannibals, or, in our case, the dead and their living conspecifics (Fox, 1975).

Consumption of dead conspecifics is widely observed across taxa, including both vertebrate (Wuensch 1986; Nishikawa et al., 2020) and invertebrate animals (Smith and Lockwood 2003; Mastrantonio et al., 2021). Despite potential risks of exposure to subsequent pathogens and predation associated with corpses, cannibalism on dead conspecifics has been reported to directly benefit animals nutritionally, for instance, increasing body mass and decreasing larval developmental period in Cuban treefrog tadpoles, *Osteopilus septentrionalis* (Babbitt and Meshaka 2000), or improving rates of

larval survival and adult emergence in tiger mosquitoes, *Aedes albopictus* (Mastrantonio et al., 2021). In termites, cannibalism plays an important role in corpse management, in which freshly killed corpses are consumed for nutrient recycling (Sun et al., 2017).

Death-related behavioral responses traced to common ancestors at or above the family level were reconstructed differently between Protostomia and Deuterostomia clades, respectively (Figure 1.4). Cannibalism was suggested as the most ancestral response to all Bilateria. Avoidance is suggested to have evolved within common ancestors of Vertebrata. Suggested origins for sexual responses, attraction, burial, corpse removal, and aggression were also reconstructed within Arthropoda. Only origins for sexual responses and carriage were suggested within Vertebrata. Among behavioral responses with suggested origins at the family level, burial was suggested in common ancestors of Termitidae, corpse removal in common ancestors of Formicidae and Apidae, and aggression in common ancestors of Vespidae.

Animals' behavioral responses to death have been widely investigated and thoroughly reviewed in social insects (see reviews, Sun et al., 2013; López-Riquelme and Fanjul-Moles, 2013; Sun et al., 2018) and mammals (see reviews, Gonçalves et al., 2018; Anderson, 2020). Ants and bees overwhelmingly perform corpse removal in response to dead conspecifics. However, due to a lack of information on related taxa, it is difficult to determine the origin of corpse removal behavior within this clade. When examining the termite clade, a total of 11 unique death-related behavioral responses (burial, cannibalism, corpse removal, sexual responses, grooming, aggression, avoidance, food aversion, sheltering, ignoring and shaking) have been recorded across 13 species. Our analysis suggests cannibalism as the ancestral state of the common ancestor of termites

and their evolutionary relatives, cockroaches (Inward et al., 2007). Therefore, given current information, it is possible that all other observed behaviors arose during the transition of termites to eusociality. Carriage showed suggested origins in the clades of Old World monkeys and cetaceans. In vertebrate animals, responses to dead conspecifics are suggested to arise in part from conflict between animate and inanimate attributes exhibited by corpses, which can elicit both fear and curiosity (Gonçalves et al., 2018). Lonsdorf et al., 2020 examined four hypotheses explaining the cause of infant corpse carrying in chimpanzees, due to either physiological and social factors such as mother-infant bond (the maternal-bond strength hypothesis and the post-parturient condition hypothesis), environmental factors such as decomposition rate (the slow decomposition hypothesis), or cognitive factors such as not recognizing the death of an infant (the unawareness of death hypothesis), but found no substantial support for any of them. Four suggested origins for sexual responses were identified, with two in vertebrate animals and two in invertebrates. In most cases, the behavior is documented as occurring in nature, but its incidence is unknown (Russell et al., 2012; Ashaharaza et al., 2020). A proximate explanation is usually suggested as the persistence of sex pheromones after death, although empirical studies in this regard are lacking (Costa et al., 2010). Benefit has been suggested with evidence of viable offspring produced from this behavior in some amphibian species (Izzo et al., 2012; Groffen et al., 2019).

Our reconstruction of death-related cues suggested the use of chemical cues as ancestral to Bilateria. An origin for the use of visual cues was suggested only in the common ancestor of most birds. For example, Western scrub-jay, *Aphelocoma californica*, respond to presentations of prostrate dead conspecifics (made of dried scrub-

jay skin and feathers) with vocalization and subsequent cacophonous aggregation but with aggressive responses to standing scrub-jay model (made by the same manners like the prostrate dead model but mounted like standing alive), indicating that visual cues related to death were detected by western scrub-jay in death recognition (Iglesias et al., 2012). Visual cues are usually considered the default for death recognition in terrestrial vertebrates (Gonçalves and Biro, 2018). However, a lack of controlled studies observing the mechanisms that trigger death-related behaviors leads to a lack of evidence that can be used to reconstruct the origins of visual or tactile cues. Our reconstruction of chemical death cues is consistent with the previous hypothesis of fatty acids as ancient death-related chemicals in Arthropoda suggested by Yao et al., 2009. Similarities in death-related behavioral response types, such as cannibalism, and cue types, primarily chemical cues, between vertebrate and invertebrate animals provide future potential for comparative studies of death responses in these disparate groups.

1.5.3 Sociality might precede changes in death-related behaviors

To test the “social complexity hypothesis” in death-related behaviors against the other two alternatives, the “precursor” and “conflict” hypotheses, we must provide evidence about 1) the relationship between social and behavioral complexity and 2) the direction of causality that if social complexity drives the complexity of communications or vice versa (Freeberg et al., 2012). However, previous studies usually tested these hypotheses for the first part as the relationship between social and communicative behavioral complexity with very little evidence for the causation. For example, as concerns communication between living individuals, social complexity was suggested to partially explain the size

of alarm call repertoire in *Marmota* rodents (Blumstein and Armitage, 1997). The relationship between group size and vocal repertoire size was confirmed in a caviomorph rodent, *Cuniculus paca* (Lima et al., 2018). Gesture repertoire size was suggested to be related to social complexity in wild East African chimpanzees, *Pan troglodytes* (Roberts and Roberts, 2020). Similarly, complex societies may require similarly complex systems for corpse management so as to manage the frequent interactions with death inherent to group living (Reggente et al., 2018, Sun et al., 2018). Eusocial insects possess complex corpse management strategies that include corpse removal, cannibalism, burial, and avoidance behaviors to balance the risks and rewards associated with death as an adaptation to enhance colony success (Sun et al., 2018).

Our results also suggest a link between sociality and death-related behavioral repertoire size as predicted by the social complexity hypothesis that social animals are associated with larger and more diverse death-related behavioral repertoires than solitary animals. From the species to family levels, the size of death-related behavioral repertoires was significantly larger in eusocial groups than in presocial and solitary groups in invertebrates and larger in presocial groups than solitary groups in vertebrates (Figure 1.6). Our correspondence analysis showed specific differences in the death-related behaviors performed between species of different sociality statuses. Cannibalism, avoidance, and aggression showed little to no association with any particular sociality status. Ignoring dead conspecifics showed a weak association with solitary invertebrate animals. The remaining behaviors examined were highly divided based on sociality status. Only one death-related behavior was strongly associated with solitary animals (sexual responses), compared to six in presocial vertebrates (attracted, food aversion,

aggregation, grooming, vocalization, and carriage) and four in eusocial invertebrates (shaking, sheltering, corpse removal, and burial).

To confirm the causation, the emergence of sociality must predate the origins of behaviors associated with presocial and eusocial animals (Freeberg et al., 2012; Ord and Garcia-Porta, 2012; Barribeau et al., 2015). Our reconstructions suggest that the origins of burial, removal and carriage behaviors all follow the emergence of either presociality or eusociality, whereas the suggested origins of sexual responses were located outside of the social clades. These results show no evidence for the alternative hypothesis that complex death-related repertoires would predate the evolution of sociality due to the failure of any potential origins of sociality-linked behaviors to be identified in common ancestors prior to the emergence of sociality. Together with the observed correlation between sociality and the size and diversity of death-related behavioral repertoire, the emergence of sociality predating the origins of these behaviors may overall suggest the “social complexity hypothesis” that sociality might play an important role proceeding the changes in animals’ death-related behavioral repertoire in both size and diversity in both vertebrate and invertebrate animals.

Be noted that a larger size of death-related behavioral repertoire in eusocial invertebrate animals has been observed at the family level than at the species level, indicating some species possess less complex death-related behaviors within this group (Figure 1.6&Table 1.3). While some eusocial groups, such as ants and termites, demonstrate a diverse array of death-related behaviors, many Hymenopteran species exclusively perform corpse removal (see review Sun and Zhou, 2013; López-Riquelme and Fanjul-Moles, 2013). Comparative studies have suggested that the complexity of

communications might not always follow the increase of social complexity in Hymenoptera, for example, the expansion of CHC and chemoreceptor repertoires were found preceding the evolution of eusociality (Kather and Martin, 2015; Zhou et al., 2015) and mushroom body investment was found to reach a peak in intermediate sociality then decrease in eusocial Hymenopterans (O'Donnell et al., 2015). The evolution of death-related behaviors may be driven by factors other than sociality (Ord and Garcia-Porta, 2012), for example, 1) habitat influencing chemical communications (Krång et al., 2012), 2) different decomposition processes between different taxa (Carter et al., 2007; Sun et al., 2017), and 3) level of death recognition, in which presocial animals, especially mammals, involving both group member and individual recognition when encountered with dead conspecifics (Gonçalves and Biro, 2018), while undertaking behaviors in eusocial insects relying on nestmate and caste recognition but no evidence for individual recognition (Sun et al., 2013; Shi et al., 2021), 4) the evolution of cognition (Gonçalves and Biro, 2018), then facilitate the evolution of sociality (Freeberg et al., 2012). Meanwhile, eusociality has evolved multiple times in Hymenoptera, the emergence and disappearance of sociality within specific taxa and their relationship with the evolution of death-related behaviors were not considered in this study (Hughes et al., 2008). Diverse behavioral repertoires might reflect specific needs regarding corpse management, including an enclosed nest environment, limited food access, colony defense against pathogens and intruders, as adaptations of eusocial animals to their ecological niches.

It would also be difficult to fully test the hypotheses in vertebrate as in invertebrate animals. To our knowledge, no record of death-related behaviors was

documented in eusocial vertebrate animals, such as naked mole rats. Complexity of death-related behavioral repertoire was only compared between solitary and presocial vertebrates in this study. Is the hypothesis we tested also supported in the evolutionary route in vertebrate animals from solitary through presocial to eusocial or the complexity would reduce during the evolution from presocial to eusocial? Evidence would be necessary to complete this part of deduction.

1.5.4 Limitations and biases

It should be noted that this review has several limitations: 1) we excluded keywords related to studies in human that may have also automatically removed related papers with specific terms in them, especially in studies highly associated with human studies, for example, those examining non-human primates. We included cases from review papers to limit this bias; 2) the origins of death-related behaviors suggested by our analysis reflect current data. With a more complete record, it is possible that these origins may be pushed further back or that new origins would be identified, the relationship between sociality and death-related behaviors could be more clarified; 3) records of death-related behaviors are likely biased towards those that are easily observed, such as corpse removal and cannibalism. Other behaviors may be more widespread than the current record suggested but are simply more difficult to observe or empirically test for their existence; and 4) given the inherent complexity of aspects such as behavior and sociality, a certain degree of simplification is necessary to perform a comparative study (Blumstein and Armitage, 1997; Freeberg et al., 2012). Considering the number of species involved in our comparative analysis, level of sociality was categorized based on the interactions within

species without considering factors such as group size, frequency and consistency of interactions, and social structures (Ord and Garcia-Porta, 2012; Blumstein and Armitage, 1997). Indicators for quantitative evaluation in both sociality and behavioral repertoire should be explicitly defined.

1.6 Summary and perspectives

Animals have evolved diverse behaviors that aid in identifying and responding to dead conspecifics. To further understand the evolution of these behaviors and their relationship with the evolution of sociality, we performed a quantitative review analyzing the evolutionary history of death-related behaviors across all animals. Our results suggest that 1) there is promising integration across the currently divided field of death-related research accompanied by a continuous increase of interest, 2) attraction to the dead in the form of cannibalism is an ancient and widely spread response to death, and 3) in general, the evolution of sociality precedes the expansion of death-related behavioral repertoires in terms of both size and diversity with exceptions in eusocial invertebrates. This study might be limited and biased due to a relatively small database. For future studies, recording death-related behaviors such as avoidance and non-direction responses would aid in reconstruction of the evolutionary histories of such behaviors. Additional data would allow origins for behaviors to potentially be dated further backwards. Better documentation of death-related behaviors in general, especially in eusocial vertebrate animals, would clarify the factors related to their evolution.

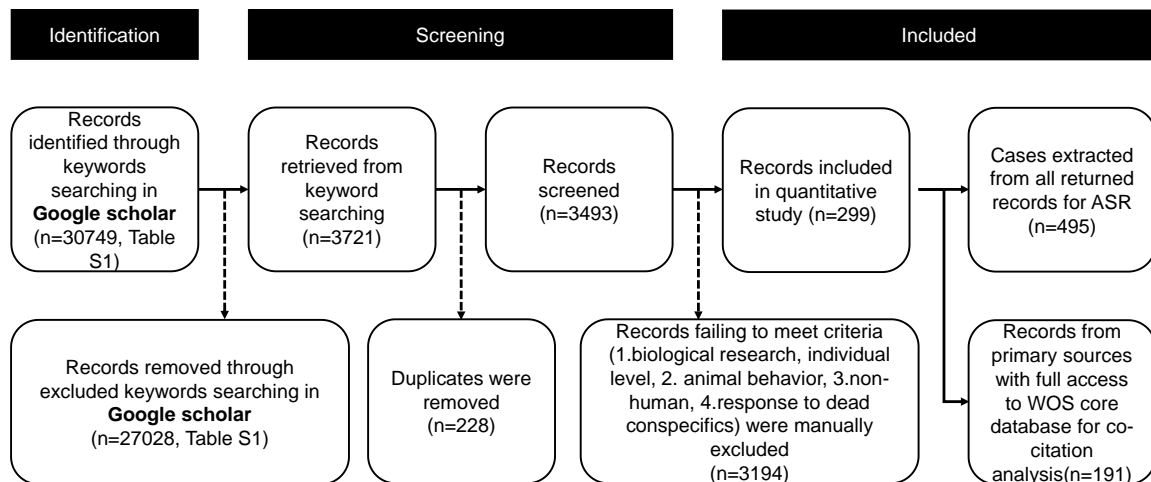


Figure 1.1 Flowchart of article selection with PRISMA.

Searching and screening process of articles and cases included in this review. Numbers of articles included or excluded in each step was marked.

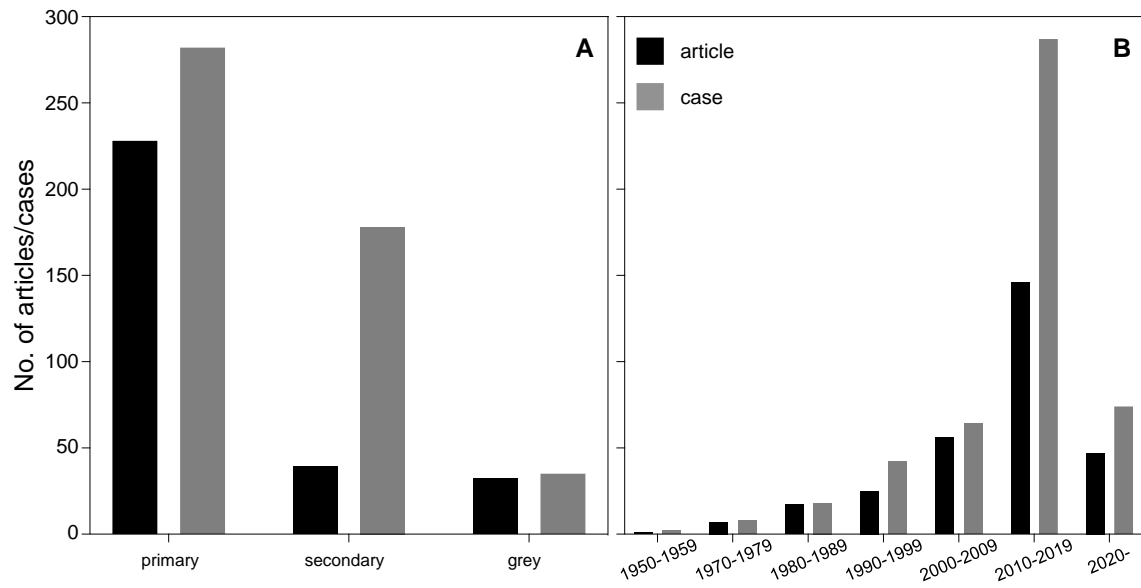


Figure 1.2 Summary of publication and case information.

Bar charts show the distribution of all included articles ($n = 299$) and cases ($n = 495$) from PRISMA as separated by (A) scholarly source type (for details, see Table S2) and (B) publishing decade.

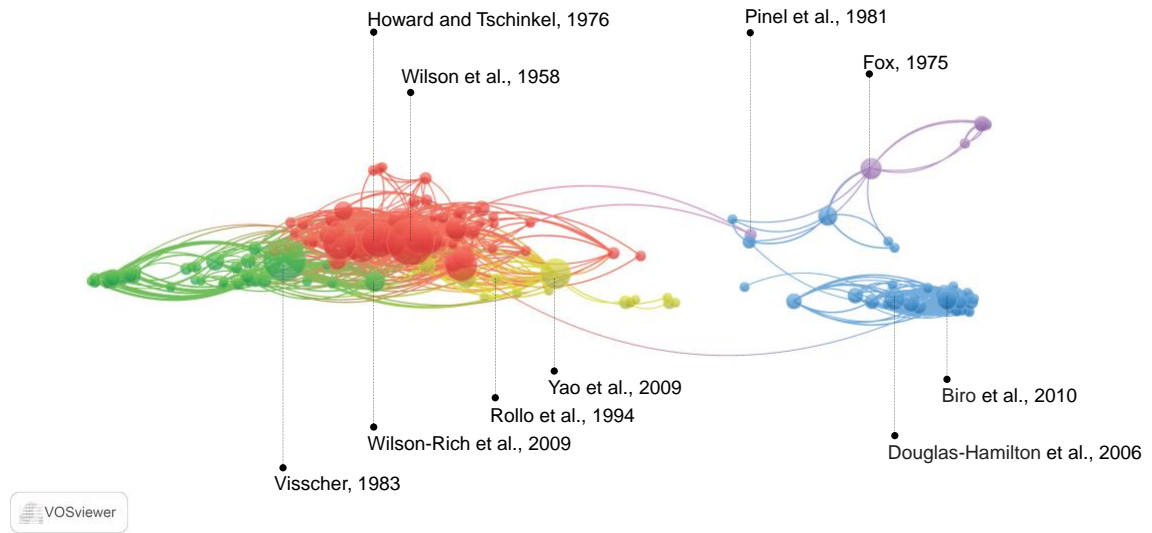


Figure 1.3 Visualization of co-citation of selected articles on death-related behaviors in non-human animals.

Nodes represent cited references (n=162). Sizes of nodes represent the citation frequency of each cited reference. Links between nodes indicate that both references were cited in the same subsequent publication. Different colors of nodes indicate different clusters representing different topics (n = 5). References with the two largest total link strength values in each cluster were labeled with author and publishing year.

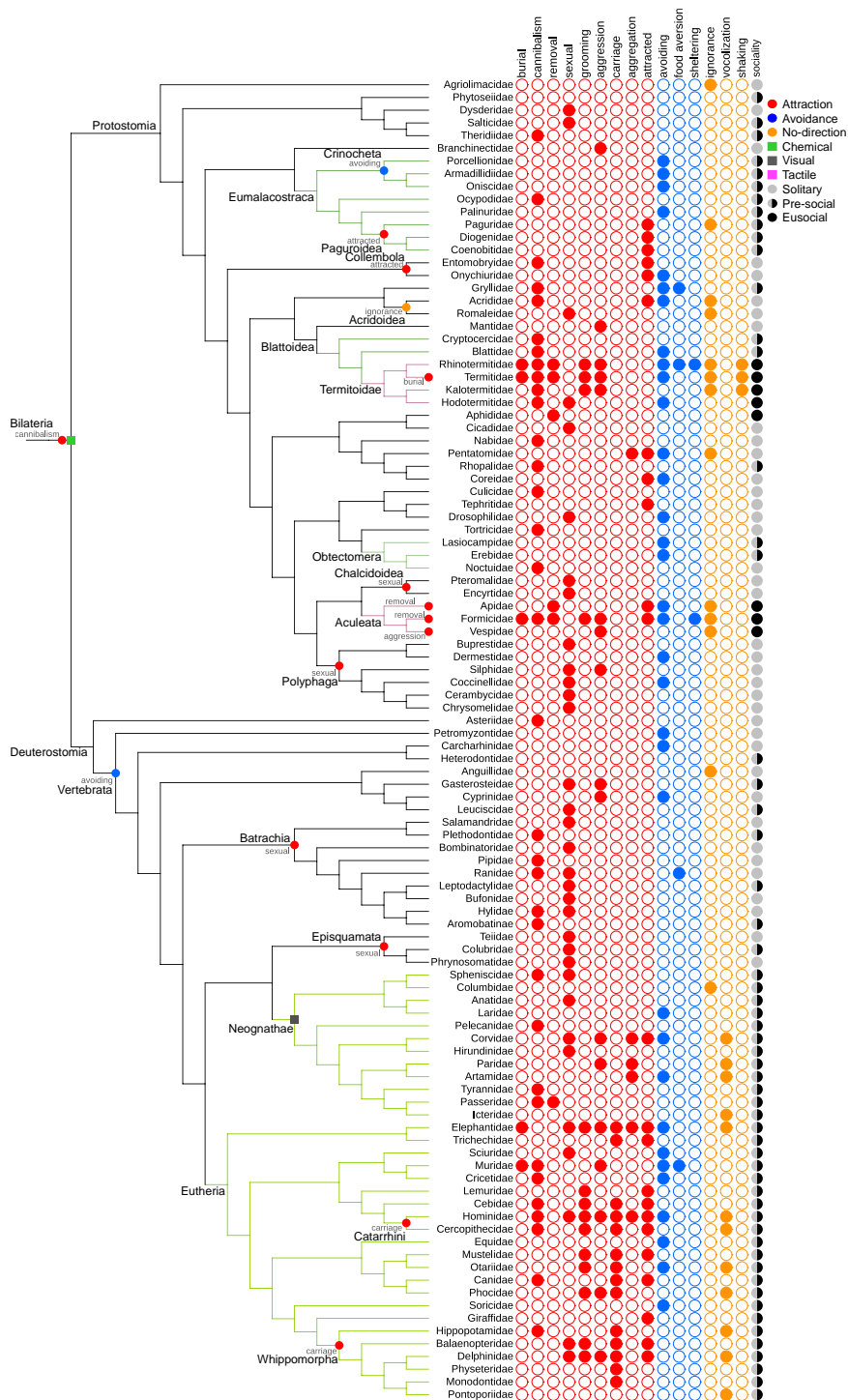


Figure 1.4 Ancestral state reconstruction of primary death-related behaviors and cues.

Three primary death-related responses (attraction, avoidance and no-direction response) and three death-related cues (chemical, tactile, visual) were reconstructed and labeled in a

phylogenetic tree with 104 families with unordered parsimony. Origins of different responses and cues by parsimony were marked as different colors. Circular shapes represent behavioral responses and rectangular shapes represent death-related cues. Presence of behaviors was shown on the right of the tree with filled shapes as “present” and unfilled shapes as “absent”. Sociality status was indicated on the far right with a grey-filled circle in solitary species, a half grey-half black circle in presocial species, and a black-filled circle in eusocial species. Sociality status within the tree was indicated by branch color, where black branches indicated solitary, light green indicated pre-social, and purple indicated eusocial. This phylogenetic tree was generated based on the NCBI taxonomy database (<https://www.ncbi.nlm.nih.gov/taxonomy>) using the online software Phylot (<https://phylot.biobyte.de/>).

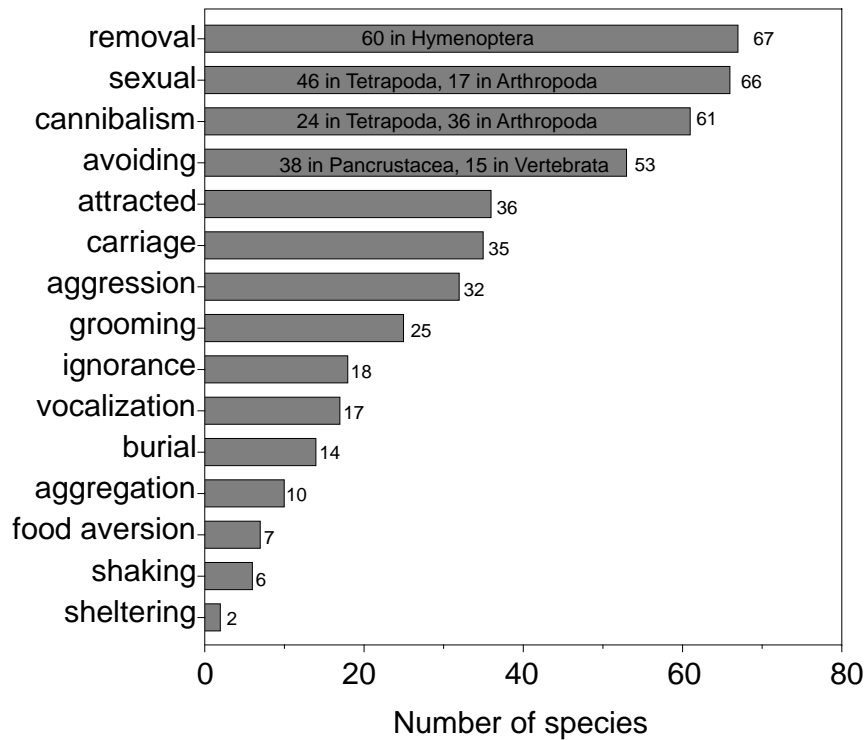


Figure 1.5 Summary of death-related behaviors at the species level.

Bar chart showing the number of species in which each death-related behavior is present. The number of species showing the behavior is labeled in each bar. Behaviors shown by more than 50 species were labeled with the largest clades exhibiting them.

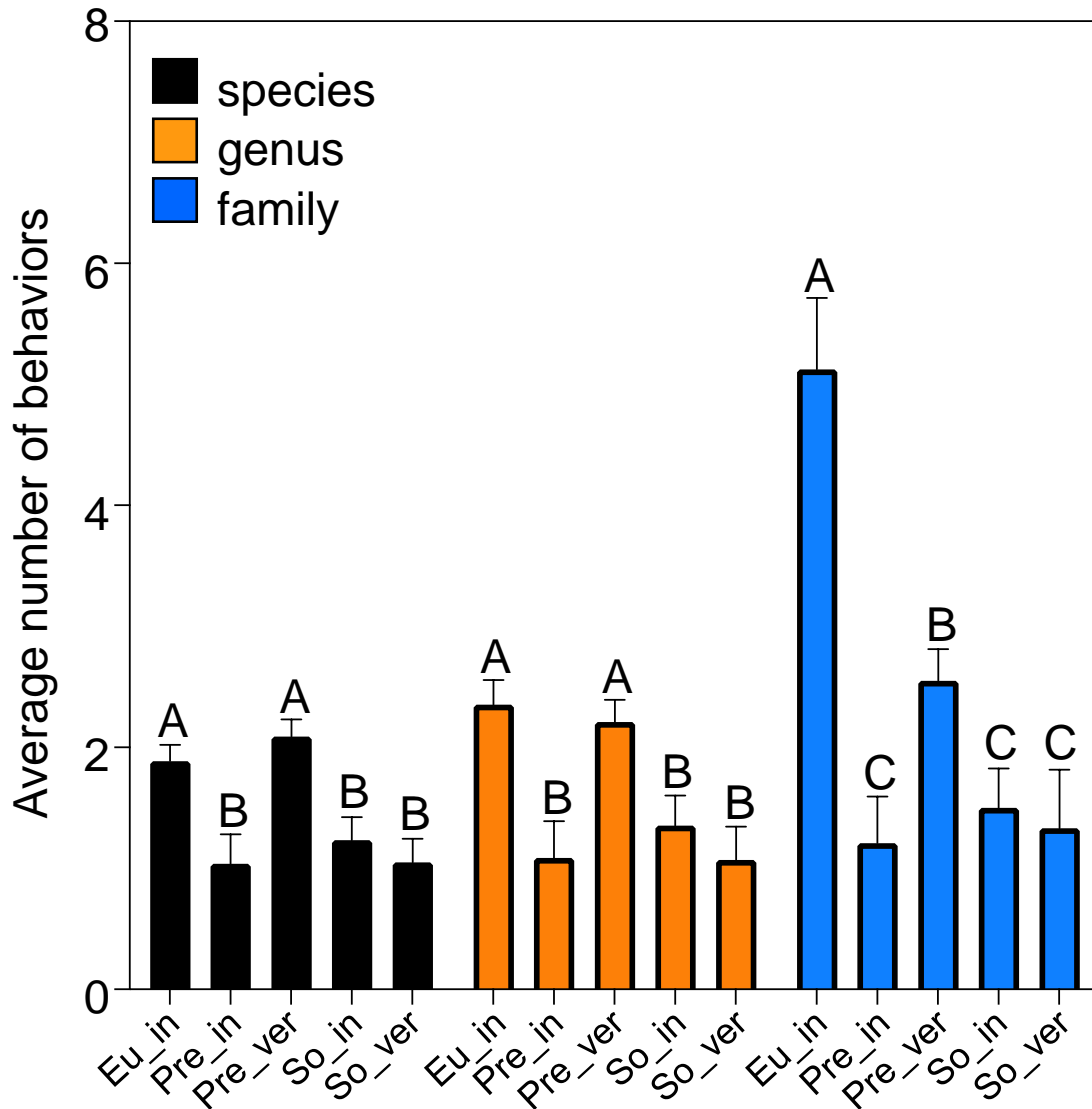


Figure 1.6 Death-related behavioral repertoires at different taxonomic levels.

The average number of behaviors exhibited by organisms of a particular sociality status. Colors indicate on which taxonomic rank the data was summarized, i.e., species (black), genus (orange) or family (blue) (Mean \pm SEM). Levels with no shared letters indicate significant differences ($p < 0.05$, one-way ANOVA). *In* represents invertebrate animals and *ver* represents vertebrate animals. *Eu* stands for eusocial; *Pre* stands for presocial and *So* stands for solitary.

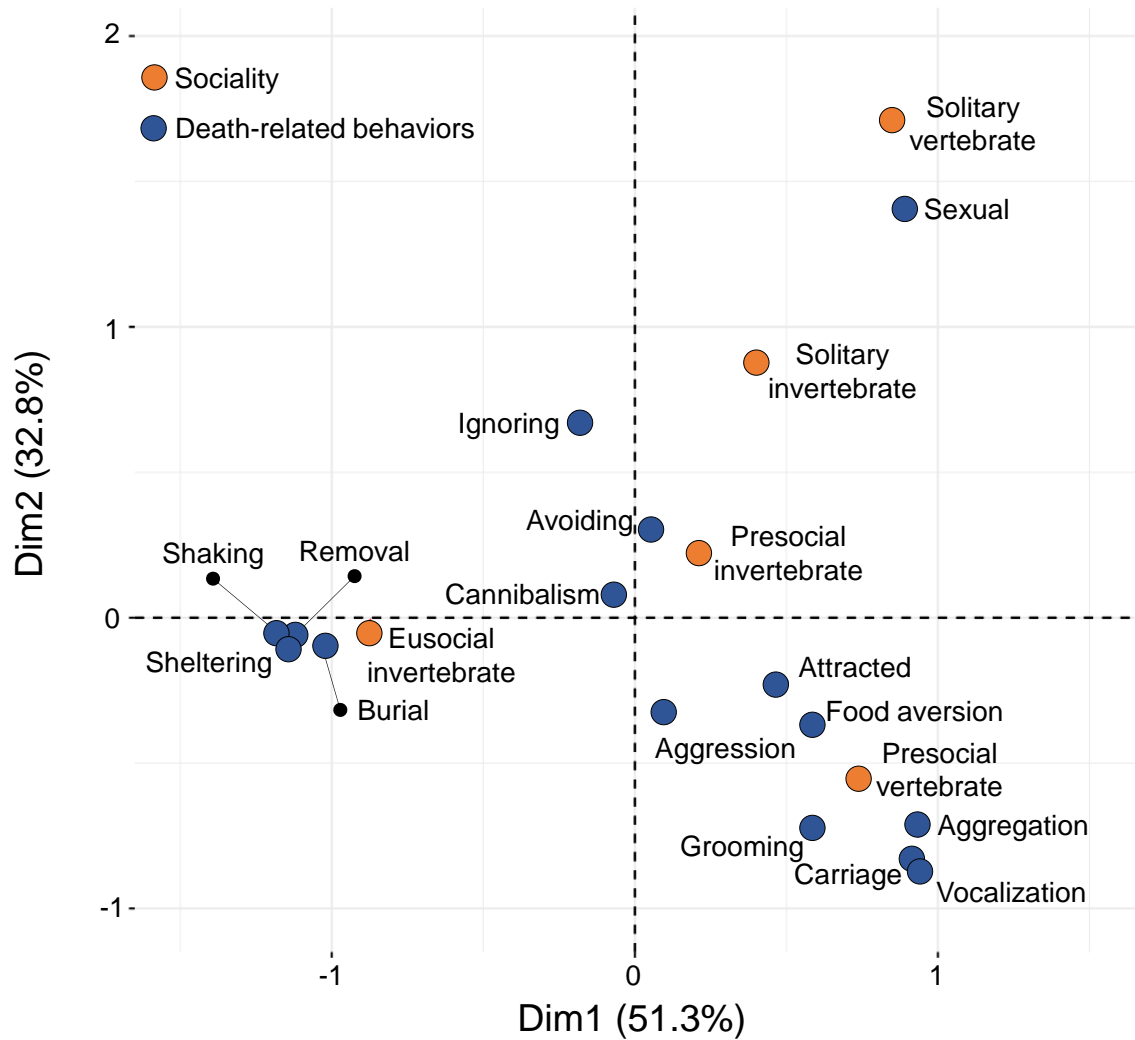


Figure 1.7 Correspondence analysis: relationship between sociality status and death-related behaviors.

Biplot representing results from the correspondence analysis with dimensions 1 (Dim1) and 2 (Dim2). Sociality status was labeled as orange and each behavior was labeled as blue. Specifically, orange circles showed five sociality status, namely eusocial invertebrate, presocial invertebrate, presocial vertebrate, solitary invertebrate and solitary vertebrate. Blue circle represented death-related behaviors, including 9 behavioral responses in attraction (burial, cannibalism, removal, sexual, grooming, aggression, carriage, aggregation, and attracted); 3 behavioral responses in avoidance (avoiding, food aversion,

and sheltering) and 3 behavioral responses in no-direction (ignoring, vocalization, and shaking).

Table 1. 1 Categories and definitions of sociality

Death-related cues/behaviors	Subcategories		Description
Cues	Chemical		chemical signals from corpse, dead tissue, crushed body were involved in death recognition
	Tactile		physical contact was involved in death recognition
	Visual		vision was involved in death recognition
Behaviors	Attraction	burial	corpse was covered with materials
		cannibalism	corpse was consumed by living conspecifics (killing then cannibalized were not included in this study)
		removal	corpse was moved away from living conspecifics
		sexual	copulation act to dead conspecifics, (sexual cannibalism is excluded)
		grooming	touching, cleaning behavior to dead conspecifics
		aggression	attacking dead conspecifics
		carriage	carrying, holding dead conspecifics (not for disposal)
		aggregation	gathering around dead conspecifics
		attracted	movement orientating to conspecific corpse or death-related cues but couldn't be categorized as above attraction response.

Avoidance	avoiding	movement away from dead conspecifics or death-related cues
	food aversion	avoiding dead conspecific consumption
	sheltering	resting/hiding under substrates or in specific area away from dead conspecifics
Non-direction	ignoring	neglecting the dead
	vocalization	vocalizing when encountering with dead conspecifics
	shaking	shaking body when encountering with dead conspecifics

Table 1. 2 Categories and definitions of death-related cues and behaviors

Sociality	Subcategories	Description
Solitary		Animals do not associate except for courtship and mating, showing none of traits of sociality
Presocial	Subsocial	Animals live in family groups consisting of parents and immature offspring with brood defense or brood provisioning by parents.
	Parasocial	1. Communal: parents share nest but care for their own offspring; 2. Quasisocial: parents share nest and cooperatively care for offspring; 3. Semisocial: parents share nest and cooperatively care for offspring, also with reproductive division of labor, but no overlapping generations.
Eusocial		1. Reproductive division of labor; 2. Overlapping generation; 3. Cooperative brood care (primitive and advanced eusociality were not distinguished here)

Table 1. 3 Number of species, genera and families in each sociality status involved in this study.

Sociality status		No. of Species	No. of Genus	No. of Family
Eusocial	Invertebrate	88	51	8
Presocial	Invertebrate	26	23	19
	Vertebrate	78	62	40
Solitary	Invertebrate	43	34	26
	Vertebrate	41	28	12

CHAPTER 2. CAUSES OF DEATH AND THEIR INFLUENCES ON CORPSE MANAGEMENT IN *RETICULITERMES FLAVIPES*

2.1 Abstract

To cope with death, termites have developed sophisticated corpse management strategies. Death caused by different factors can present different types of risks. How termites respond to these dead nestmates is largely unknown. To address this question, we used the eastern subterranean termite, *Reticulitermes flavipes*, the most widely distributed termite species in North America, as our model. Based on previous studies and our preliminary observation, we hypothesized that termites respond differently to nestmate corpses resulting from different causes of death.

To examine this overarching hypothesis, we 1) generated termite corpses using both chemical control agents and naturally occurring environmental factors; 2) examined postmortem chemical changes in these corpses; and finally, 3) documented the subsequent behavioral responses of nestmates to these corpses. Our combined results suggested that: 1) *R. flavipes* workers cannot distinguish individuals killed by termiticides from those killed by naturally occurring abiotic factors; 2) death by biotic factors accelerated the release of late death cues and the deployment of burial behavior in nestmate workers; and 3) the quantitative and qualitative changes in postmortem chemical profiles account for the observed differential behavioral responses.

This study not only contributes to our overall understanding of how eusocial insects deal with nestmate corpses, but also sheds light on the basis underlying behavioral resistance to chemical controls in a devastating subterranean termite.

2.2 Introduction

Termites have evolved complex corpse management strategies to deal with dead conspecifics, including avoidance, cannibalism and burial, which mitigate death-related risks and benefits (See reviews: Sun et al., 2013; Sun et al., 2018). Because termites feed on a low-nitrogen diet, cannibalizing dead conspecifics can benefit them from nutrient recycling (Hungate, 1941) and possibly symbionts acquisition (Thorne, 1990).

Meanwhile, cannibalism on nestmates killed by entomopathogenic fungi or severely diseased individuals lowers the likelihood of epizootic by inhibiting the ingested fungi through antifungal activity in the gut (Chouvenc et al., 2009 and 2010; Chouvenc and Su, 2012; Davis et al., 2018). Cannibalism resorts to burial behavior when the risks posed by corpses are too great, such as when the corpses are severely decomposed, heavily infected by entomopathogens, or in vast quantities (Neoh et al., 2012; Sun et al., 2017; Shi et al., 2021; Chouvenc and Su, 2012). Even if burial is more labor-intensive and results in the loss of nutritional value compared to cannibalism, it effectively isolates healthy individuals from potential risks associated with the dead, reducing further disease transmission and competitive threats. Avoidance in subterranean termites is similar to burial and often involves sealing off the tunnels to prevent other termites from approaching the region with corpses who have been infected with pathogens, killed by insecticides, or generated from competition (Su et al., 1982; Su, 2005; Li et al., 2010). The various corpse management strategies in termites strike a balance between the nutritional benefits and risks associated with dead colony members.

Termite workers perform a wide range of risky tasks, including foraging, feeding, brood care, nest maintenance and colony defense (Bignell et al. 2010). As a result, they are frequently exposed to a variety of naturally occurring environmental factors that

might result in death. Death caused by these factors could in turn present different types of risks to the colony. Naturally occurring environmental factors could be classified as abiotic and biotic (see review in Sun et al., 2018). Specifically, corpses killed by abiotic environmental factors, such as extreme temperature (freezing or heat), and desiccation, might indicate a risk of exposure to extreme climate conditions (Mellanby, 1939; Ratnayake et al., 2019). Meanwhile, corpses can be killed by biotic environmental factors, such as pathogens, predation and competition. A corpse killed by pathogens can potentially spread infectious agents, such as entomopathogenic fungi, bacteria, viruses, protozoans, and parasitic worms (Chouvenc et al., 2011). Termites can also die from direct and indirect antagonistic interactions with predators and competitors, which could present risks of conflict with nearby predators or competitors (Li et al., 2010; Tian et al., 2017). Termites may exploit different behavioral responses toward dead conspecifics killed by these factors. In two subterranean termite species, *Coptotermes formosanus* and *Reticulitermes speratus*, heat-killed corpses induced longer investigation time with higher frequency than corpses killed by crushing (Neoh et al., 2012). *Reticulitermes flavipes* workers groomed individuals infested with an entomopathogenic fungus, *Metarhizium anisopliae*, more frequently than healthy ones and cannibalized them when they got visibly diseased (Davis et al., 2018). When *R. virginicus*, was killed by *M. anisopliae*, fungus-killed corpses were not cannibalized, as those killed with chloroform, but buried (Kramm et al., 1982). In *C. formosanus* and *C. gestroi*, termite corpses generated from interspecific competition elicit sand deposition and subsequent tunnel blockages (Li et al., 2010). Corpses killed by these factors pose both benefits of nutritional sources and risks associated with death. Since risks changes with different causes of death, behavioral

plasticity in managing these corpses would be advantageous to balance the benefits and risks.

Besides the naturally occurring factors, another particular cause of death for termites is the chemical control agent. Over 3100 termite species have been described, of which 183 cause damage to human buildings (Edwards and Mill, 1986; Krishna et al., 2013). Most of these 183 species are subterranean termites. To manage subterranean termite infestations, we have predominantly relied on chemical treatments (Su and Scheffrahn, 1998; Verma et al., 2009). Barrier treatment is a traditional approach that has been widely utilized in the pest control industry for decades (Su and Scheffrahn, 1998). This approach aims to apply a liquid pesticide to the soil underneath and surrounding a building to create a long-lasting chemical barrier in the soil that prevents termites from penetrating and infesting the structure. Non-repellent pesticides with delayed toxicity, for example, fipronil as in Termidor® (BASF, NC, US), imidacloprid as in Premise® (Bayer, MO, US), have been suggested as the most commonly used and reliable active ingredients in this approach killing the termites when they tunnel through the treated soil (Parman and Vargo, 2010; Potter, 2011; Vargo and Parman, 2012). Both fipronil and imidacloprid are neurotoxins of insects. Fipronil is a phenyl pyrazole insecticide that kills the termites by blocking the GABA-regulated chloride channels and causing hyper-excitation in the central nervous system, whereas imidacloprid belongs to the neonicotinoids and blocks nicotinic acetylcholine receptors, resulting in paralysis and death (Narahashi et al., 2007; Zhang et al., 2000). When incorporated into the soil, fipronil degrades with a half-life ranging from 93 to 226 days, compared to 26.5 to 229 days for imidacloprid (Tingle et al., 2003; Fossen, 2006). For commercial soil treatments,

their effects can remain for years to provide a continuous effect in preventing termites from invading buildings (Wagner et al., 2002). Fipronil and imidacloprid use has been heavily restricted in agriculture in the US and EU due to concerns about their acute toxicity on other non-target arthropods, particularly pollinators like honeybees (Holder et al., 2018; Suchail et al., 2001). They can only be used to a limited extent in urban structural pest control and pet products. (Simon-Delso et al., 2015; Van der Sluijs et al., 2015). Another important strategy is the use of termite baits, which combine cellulose with a slow-acting, non-repellent insecticide, for example, insect growth regulators, which interfere with termite development (Su and Scheffrahn, 1998; Potter, 2011). Termites consume the bait and distribute it to other termites in their nest, which causes a gradual decline in the termite population. Chitin synthesis inhibitors are one of the most important classes of insect growth regulators. For example, hexaflumuron has been found to inhibit ecdysis in *Reticulitermes* and *Coptotermes* termites and is effective against field termite colonies (Su and Scheffrahn, 1993 and 1998; Su, 1994). Affected termites cannot successfully shed their exuviae during their subsequent molting, which results in death from either cannibalism by other nestmates or dark necrotic lesions (Su and Scheffrahn, 1993). The delayed toxicity allows the transfer of hexaflumuron throughout the colony by food distribution or cannibalism. Workers can avoid or seal off the tunnel entering the area with corpses killed by these chemical control agents (Su, 1994 and 2005) or cannibalize them (Su and Scheffrahn, 1993). Understanding how these chemical control agents alter the undertaking behaviors of termites is important to better comprehend the impact of insecticides and improve their evaluation in termite control (Su et al., 1982).

These behavioral responses toward dead conspecifics in termites are primarily mediated by chemical death cues (See reviews: Sun et al., 2013; Sun et al., 2018). The accumulation of fatty acids during the decomposition process, along with phenol and indole, is responsible for inducing burial behavior in termites (Chouvenc et al., 2012). In the eastern subterranean termite, *Reticulitermes flavipes*, chemical profile and undertaking behavioral patterns have been well established in freezing-killed corpses (Sun et al., 2017). 3-Octanol and 3-octanone, are named as early death cues and found to be released right after death and utilized by workers to locate corpses. The early death cues gradually diminish during decomposition as the amounts of late death cues (phenol, indole, and fatty acids) increase. Termites may switch from cannibalism to burial of corpses due to the interplay of these death-related chemicals to balance the nutrition benefits and risks associated with corpses (Sun et al., 2017). Fatty acids in dead bodies are suggested to be generated from the enzymatic hydrolysis of triglycerides (Blum, 1970; Akino and Yamaoka, 1996). Although the synthesis of 3-octanol and 3-octanone is barely investigated in termites, the synthesis of C8 volatiles is suggested to be an enzyme-catalyzed process of fatty acids in plants and fungi, implying that the synthesis of the early death cues in *R. flavipes* is most likely enzymatic (Kihara et al., 2014; Combet et al., 2006). In *R. flavipes*, oleic acid stored in triglycerides has been suggested as a major source of energy during stress (Mauldin, 1977). Stress during different modes of killing can affect the contents of death-related chemicals on termite bodies, change the chemical profiles of corpses, and subsequently alter how other termites behave toward them. Heat and desiccation could reduce the enzyme activity during decomposition and decrease the production of lipid enzymatic products (Koelmel et al., 2018; Rupley and

Careri, 1991). Fungal infection has been suggested to accelerate the accumulation of oleic acid in dead pupae in the fire ant, *S. invicta* (Qiu et al., 2015). Intraspecific competitive stress has been suggested to cause strong behavioral and physiological reactions with increased energy costs, which may demand oleic acid as a backup energy supply, influencing the amount of oleic acid on the corpses (Tian et al., 2015; Mauldin, 1977). For chemical control agents, the repellency of the insecticides themselves and/or death-related chemicals can have an impact on termite behaviors (Su et al., 1982). These chemical control agents, fipronil, imidacloprid and hexaflumuron, are considered non-repellent to termites (Remmen and Su, 2005; Mao et al., 2011; Su et al., 1987; Vahabzadeh et al., 2007). How they influence the release of death-related chemicals is still unclear. Meanwhile, chemical control agents such as fipronil and imidacloprid contain contact toxicity, may negatively influence the mobility of termites and in turn impact undertaking behaviors (Su, 2005; Zhang et al., 2000). It is still uncertain how different causes of death alter the chemical profile of the corpse or whether termites may detect the differences and adapt their behavior accordingly.

In this study, we aimed to investigate how termites respond to different causes of death. The eastern subterranean termite, *Reticulitermes flavipes*, one of the most widespread termites in North America, and one that is invasive in Europe (Evans et al., 2013; Bourguignon et al., 2016), was used as the model animal in this study. Freezing, heat, and desiccation were selected as naturally occurring abiotic factors, whereas fungal infestation and indirect intraspecific competition (hereafter: competition) were selected as biotic factors. For chemical control agents, three most commonly used chemicals in termite control were chosen. Two neurotoxins, fipronil and imidacloprid, were selected

because of their non-repellency and common usage in barrier treatment. One insect chitin synthesis inhibitor for baiting stations, hexaflumuron, was also selected. Based on previous studies and our preliminary observations, we hypothesized that termites respond differently to dead conspecifics killed by different causes. To test this hypothesis, we have conducted the following objectives: 1) generated termite corpses using naturally occurring abiotic and biotic factors and chemical control agents; 2) profiled the temporal changes in death-related chemicals of these corpses with different postmortem times; and finally 3) documented the subsequent behavioral responses of living individuals toward these corpses.

2.3 Material and methods

2.3.1 Overall experimental design

In laboratory conditions, eight modes of killing (three synthetic pesticides: fipronil, imidacloprid, and hexaflumuron; three abiotic factors: freezing, heat, and desiccation; and two biotic factors: competition stress and fungal infestation) were selected to simulate potential causes of death of *Reticulitermes flavipes* in nature. For comparisons, killing and corpse collection techniques were chosen while taking into account the consistency of corpse status, i.e., integrity, temperature, and precision of the point of death. Corpses killed by these factors were allowed to decompose for a series of postmortem times. Chemical analysis was performed on these corpses to investigate if postmortem release patterns of death-related chemicals were influenced by these factors. In addition, living nestmates were challenged with these corpses to examine behavioral responses toward these corpses (Figure 2.1A).

2.3.2 Termite colony collection and maintenance

Two field colonies of *R. flavipes* were collected from Daniel Boone National Forest (Wolfe, KY) and another was collected from the Arboretum, State Botanical Garden of Kentucky, at the University of Kentucky (Lexington, KY). Termites were captured by placing cardboard rolls under rotten fallen wood as traps for one to two weeks. Cardboard rolls were collected and taken back to the lab and termites were extracted from these traps. Newly collected termites were isolated in a Petri dish lined with a watered paper towel for at least 14 days, then transferred into a cubic plastic container (L: 31.3 cm, W: 23.0 cm, H: 10.2 cm, Pioneer Plastics, Inc., KY, US) filled with moistened mulch and stacked pinewood boards. This step was repeated for termites collected from the same trunk for the same collecting season. Shoeboxes with collected termites were stored in an incubator in 24h darkness ($27 \pm 1^\circ\text{C}$, ~90% RH within boxes). Termites were used in this study within six months of collection.

2.3.3 Corpse preparation

Naturally occurring environmental factors

Abiotic factors

Low temperature, high temperature, and low humidity treatments were selected to simulate potential abiotic causes of death (freezing, heat, and desiccation) in *R. flavipes*. For freezing, termites were placed in an ultra-low temperature freezer (-80°C) for one minute, then thawed at room temperature for five minutes before postmortem time treatment (Sun et al., 2017). This choice of temperature was selected to create an acute time point of death with a sacrifice of ecological relevance. Frozen corpses were thawed

at room temperature to limit the influence of low temperature on chemical detection and behavioral responses and to maintain consistency of corpse temperature with corpses generated through other means.

For heat treatment, 44°C was selected as the treatment temperature based on the critical thermal maximum (temperature at which motor control is lost and death might occur in animals) of *R. flavipes* (43.5-44.9°C, Hu and Appel, 2004). Treatment time was determined with a preliminary test to find the shortest killing time at 44°C (25 min). Ten workers were put in a Petri dish (diameter = 3.5 cm) lined with one layer of unbleached paper towel treated with 60 µL of water to prevent desiccation. Dishes were sealed with parafilm and placed in an oven (44°C) for 25 min to kill the termites.

Termites are highly susceptible to desiccation. In a previous study, 0% relative humidity (RH) caused 50% mortality in around 24h in an *R. flavipes* colony with 100 individuals (Janowiecki and Vargo, 2021). In our study, 10 workers were placed in Petri dishes (d = 3.5 cm) with no lids, paper or water supplied, then stored in an incubator (27 ± 1°C, 30% RH). The treatment time (24h) was determined with a preliminary test using a series of treatment times (20, 21, 22, 23, 24, 25h), in which termites were found 100% immobilized but still alive at 23h and over 90% mortality was observed at 24h. Mortality was not assessed during each treatment to prevent disturbances in humidity and temperature. Untreated living workers were used as controls for freezing, heat and desiccation.

Biotic factors

Under natural conditions, *R. flavipes* workers might frequently die by either directly defending the colony against pathogens, predators, and competitors (Sun et al., 2018), or indirectly due to the presence of predators or interspecific/conspecific competitors (Tian et al., 2017). In order to preserve the integrity of corpses, fungal infestation and contactless competitive stress from intercolonial conspecifics were chosen as biotic causes of death.

Metarhizium is a common entomopathogenic fungal genus in nature, which kills its host by penetrating the cuticle, multiplying inside the body cavity, disrupting the host's immune system, and producing secondary metabolites (Lu and St leger, 2016; Vivekanandhan et al., 2020). For fungal inoculation, a wildtype strain of one species from this genus, *Metarhizium robertsii*, was used. A conidia suspension was prepared with 0.05% Tween 80 and diluted to 10^7 conidia/ml (based on a preliminary test on virulence). 100 μ L suspension was applied to a piece of unbleached paper towel that was lining the bottom of a plastic Petri dish ($d = 3.5$ cm). Twenty *R. flavipes* workers were exposed to the conidia by freely walking on the treated paper towel. 0.05% Tween 80 was used as a control. Petri dishes were stored in an incubator ($27 \pm 1^\circ\text{C}$, $\sim 90\%$ RH, L:D = 0:24) for 3 days before corpse collection. Dead bodies were removed daily before corpse collection.

The set-up for generating corpses from competition stress between conspecific non-nestmates followed a survey design by Tian et al., 2017. Slits were cut into the walls of Petri dishes with 3.5 cm diameter at even intervals, for a total of 16 slits per Petri dish (width of cuts = 1 mm). Slits allow chemical transmission and antennal interaction but

prevent lethal contact. The 3.5 cm Petri dish (inner) was then placed at the center of a 5.5 cm Petri dish (outer). Twenty *R. flavipes* workers were transferred into the 3.5 cm Petri dish for a 24-hour acclimation period. After that, 40 conspecific non-nestmate workers were transferred to the 5.5 cm Petri dish. Groups for both the inner and outer Petri dishes were selected from one of three field colonies of IDs A45, R5, and RII-3. Set-ups were kept in an incubator ($27 \pm 1^\circ\text{C}$, ~90% RH, L:D = 0:24) for 3 days before corpse collection. Fifteen replicates were made for each combination of colonies to harvest corpses. Conspecific nestmate workers were used in the outer Petri dish as a control.

Chemical control agents

Three chemical control agents were selected from two main termite control approaches: fipronil and imidacloprid, which are used in barrier treatment; and hexaflumuron, which is used in termite baits (Figure 2.1B). Fipronil, a phenyl pyrazole insecticide, kills termites by blocking GABA-regulated chloride channels and inducing hyper-excitation in the central nervous system, whereas imidacloprid, a neonicotinoid, disrupts nicotinic acetylcholine receptors, producing paralysis and subsequent death. (Narahashi et al., 2007; Zhang et al., 2000). Both chemicals are non-repellent and lethal to *R. flavipes* (Remmen and Su, 2005; Gahlhoff and Koehler, 2001; Mao et al., 2011). Hexaflumuron is a chitin synthesis inhibitor belonging to the family of benzoylphenyl ureas (Hajjar and Casida, 1978), inducing ecdysis inhibition in termites and causing death during their next molting (Su et al., 1993). All chemicals used were technical grade: fipronil ($\geq 95.0\%$), imidacloprid ($\geq 98.0\%$), and hexaflumuron ($\geq 98.0\%$) (Sigma-Aldrich, St. Louis, MO, USA). Distilled acetone was used as the solvent for all three chemicals.

In the preliminary assay, a series of concentrations of acetone solutions of fipronil (0.001%, 0.01%, 0.1%, w/w) and imidacloprid (0.004, 0.02, 0.1, 0.5%, w/w) were tested by using a topical application method on *R. flavipes* workers to determine dose and treatment time. A dose of 0.5 μ L of the acetone solution of either fipronil or imidacloprid was topically applied onto the pronotum of an *R. flavipes* worker using a repeating dispenser equipped with a syringe. Treated termites were transferred into a Petri dish lined with a moistened paper towel and kept in a $27 \pm 1^\circ\text{C}$ incubator. Mortality was observed every hour. Time points of 8h for 0.01% fipronil and 16h for 0.5% imidacloprid were found to yield the highest increases in mortality and were selected as the treatment concentrations and times for corpse collection in this study. Control workers were treated with the same amount of acetone.

For hexaflumuron, a dietary application was adopted. Treatment dose and time were selected based on Su and Scheffrahn, 1993. Paper disks (diameter = 3.5 cm) made of unbleached paper towels were treated with 200 μ L hexaflumuron acetone solution (0.01%, w/w) and dried in a fume hood for 40 min. Fifty workers were transferred into a Petri dish lined with four layers of treated paper disks with 200 μ L distilled water. Treated workers were kept in an incubator ($27 \pm 1^\circ\text{C}$, ~90% RH, L:D = 0:24) for three weeks. Control units were treated with acetone only. Dead bodies were removed every day before corpse collection.

Corpse collection

In this study, death was practically defined as no coordinated movement in response to the stimulus of a fine brush. With different killing methods, the mode of action affects

how long it takes for termite workers to die. To get a more exact time point of death, termite corpses were collected using different strategies across different killing methods. Specifically, death by freezing (1 min) and heat (25 min) occurred quickly with high mortality within treatment groups. Corpses were collected immediately after the treatments. For termites killed with fipronil (8h), imidacloprid (16h), and desiccation (24h), corpses were collected every hour after the treatment period began. Death by fungi, conspecific competition (3 days), and hexaflumuron (3 weeks) took much longer than the other treatments. For these treatment groups, termites were observed and corpses were collected every two hours after the treatment period began for three days (12 hours a day). The collection time was defined as 0h postmortem for each treatment.

Postmortem treatment

All collected corpses were individually contained in a 3.5 cm unsealed Petri dish and provisioned in an incubator ($27 \pm 1^{\circ}\text{C}$, ~90% RH, L:D = 0:24) for a series of postmortem times before the behavioral and chemical experiments. In *R. flavipes*, the release and reduction of early death cues occur rapidly within 16h after death, whereas it takes more than 32h for the significant accumulation of late death volatiles and fatty acids. In addition, behavioral responses were found to be similar toward corpses with postmortem times less than 32h (Sun et al., 2017). To capture the temporal release pattern of death-related chemicals, postmortem times in this study were set at 0, 0.5, 1, 2, 4, 8, 16, 32 and 64h. In behavioral assays, they were 0, 1, 2, 4, 8, 16, 32 and 64h.

2.3.4 Postmortem chemical profile of corpses killed by different causes of death

To profile death-related chemicals, termite corpses with different causes of death were collected across a series of postmortem times (0, 0.5, 1, 2, 4, 8, 16, 32, 64h) and examined via SPME/GC-MS (Solid Phase Microextraction/Gas Chromatography-Mass Spectrometry) for the existence of death-related volatile organic compounds (VOCs) and via hexane extraction/GC-MS for the quantification of death-related chemicals. For each cause of death, three different termite colonies with three technical replications per colony were used.

To extract death-related VOCs, a 100 μm Polydimethylsiloxane phase SPME fiber (Agilent Technologies, Santa Clara, CA, USA) was injected into a glass vial (2 mL) containing 15 corpses and sealed with an 11 mm aluminum crimp cap (septa material: PTFE/red rubber) (Agilent Technologies, Santa Clara, CA, USA). Fiber was extended onto the headspace of the corpses without contact. After 15 min extraction, the fiber was injected into the GC for 1 min as a sample for GC-MS analysis. Hexane extraction was used to quantify the VOCs detected by SPME. Two hundred μL distilled hexane was added to 15 corpses inside the 2 mL glass vial with a C19 hydrocarbon as internal standard (n-nonadecane, 10 ng/ μL). Two μL of the solution was sampled from the 10 min extraction and injected into the GC-MS. For fungus-killed corpses, hexane extraction of a control suspension of *M. robertsii* did not contain either of the early death cues in our preliminary trial.

Temporal changes in oleic acid abundance were used as an indicator of accumulation of death-related fatty acids while worker corpses aged. Ten corpses were extracted for 10 min with 300 μL of distilled hexane with 10 ng/ μL n-nonadecane as an

internal standard. Supernatants were carefully transferred into a new vial and 200 μ L BF3-methanol (10%, w/w) was added for a 10 min reaction at 60°C. Two μ L of reaction solution were injected into the GC as a sample to quantify oleic acid using related methyl ester (Methyl cis-9-octadecenoate).

A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) in split-less mode with a 30 m \times 0.25 mm \times 0.25 μ m DB-5 capillary column was used for chemical profiling with helium as carrier gas. Column temperature was set to start at 40°C, hold for 2 min, and then raise to 320°C at 10°C/min. The temperature of the injection port was set as 280°C. An Agilent Technologies 5975 mass spectrometer (MS) was coupled with GC using the National Institute of Standards and Technology/National Institutes of Health/Environmental Protection Agency Mass Spectral Library for compound identification.

2.3.5 Behavioral assay with corpses resulted from different causes of death at different postmortem times

Corpses of specific postmortem times (0, 1, 2, 4, 8, 16, 32 and 64h) were individually introduced into a two-dish assay to test undertaking responses from conspecific nestmates for all eight different causes of death. Three colonies with 10 replications each were tested. The two-dish set-ups were made by connecting a living chamber (a 5.5 cm Petri dish) to a testing chamber (a 3.5 cm Petri dish) with a 3.5 cm long plastic tube (diameter = 7 mm). Both chambers were supplied with unbleached paper towels treated with 200 μ L distilled water. Sand (1 g) moistened with 150 μ L distilled water was provided in the living chamber for burial use. Twenty-nine workers and one soldier were transferred into

the living chamber for a three-day acclimation period before the start of the assay.

Corpses were individually introduced into the testing chamber through a 0.5 cm entry port on the lid. Behavioral responses towards each corpse were recorded with a Canon VIXIA HF G10 camcorder for 24 hours. The disposition of the corpses was defined as either burial or cannibalism. Specifically, if the sand was deposited onto the corpse, its disposition was defined as burial. If the corpse was moved into the plastic tube, its disposition was defined as cannibalism. The retrieval time was recorded as the time at which the corpse was moved into the entrance of the living chamber.

A single-dish assay was used to estimate the contact frequency towards each corpse.

Corpses generated through each cause of death and at different postmortem times (0, 1, 2, 4, 8, 16, 32, 64h) were tested in 3 colonies with 5 replicates each. Workers ($n = 29$) with one soldier were placed in a 5.5 cm Petri dish lined with a moistened paper towel and given a three-day acclimation period. Prepared corpses were introduced individually into the Petri dish through a 0.5 cm entry port. Behavioral responses toward each corpse were recorded with a Canon camcorder. Physical contacts between living workers and the corpse lasting longer than 10 seconds were observed and recorded for 5 min at 0, 15, 30, 45, and 60 min after the introduction of the corpse. The number of contacts was defined as the sum of the counts of contacts at these time points.

2.3.6 Data analysis

All statistical analyses were conducted using JMP 14.0 (SAS Institute Inc., Cary, NC, USA). A multiple correspondence analysis (MCA) was performed to map potential relationships between causes of death and three death-related characteristics, i.e.,

disposition, retrieval time, and number of contacts. The eight causes of death, including fipronil, imidacloprid, hexaflumuron, frozen, heat, desiccation, competition, and fungi, were involved in this MCA. Disposition was categorized as either burial or cannibalism. Numeric variables, retrieval time, and contact numbers were practically divided into categories to fit in the MCA. The categorization was conducted according to their quartile distribution to achieve four equal-size bins for each variable. Boundaries of the bins were taken as the nearest whole number. Specifically, cutoff points for retrieval time were determined as 10, 20 and 40 min (< 10 min, between 10 and 20 min, between 20 and 40 min, >40min), whereas the number of contacts was separated into thresholds of 25, 35, and 45 (< 25, between 25 and 35, between 35 and 45, > 45). A chi-square test was conducted to determine the significance of association between causes of death and each category of death-related characteristic.

Death-related behavioral characteristics (disposition, retrieval time and number of contacts) and the amounts of death-related chemicals (VOCs: 3-octanol, 3-octanone, phenol, and indole) were then compared between causes of death across postmortem time points. Disposition was represented by burial ratio, which was calculated as the percentage of corpses buried for each combination of cause of death and postmortem time point. Normality was tested for in burial ratio, continuous data of retrieval time and number of contacts, and quantities of death-related VOCs and oleic acid using the Shapiro-Wilk W test. Quantities of death-related VOCs were log-transformed, retrieval times were $\log(x+1)$ transformed, and contact numbers were square root transformed to meet the assumptions of parametric tests. A Pearson correlation coefficient was computed to assess the linear relationship between retrieval times and contact numbers. To compare

the effects of different causes of death and postmortem times and their interactions on death-related chemicals and behaviors, ANOVA was conducted. Then, a two-way ANOVA was used to compare the above variables across different causes of death and colonies and their interactions at each postmortem time followed by comparisons for all pairs using a Tukey-Kramer HSD test. To compare the amounts of oleic acid and burial ratio across different postmortem time points under different causes of death, a Wilcoxon/Kruskal-Wallis Test (Rank Sums) followed by Wilcoxon Method for non-parametric comparisons for pairs was conducted. Figures were generated using Prism 9 for macOS (GraphPad Software, San Diego, CA).

2.4 Results

2.4.1 Postmortem changes in chemical profiles of corpses killed by different causes
Chemical profiles showed differences in quantities and release patterns of death-related chemicals across different causes of death (Figures 2.4 and 2.5). Early death cues were detected in all types of corpses before 1h postmortem and vanished afterwards. Phenol and indole could not be detected until 16h postmortem in corpses killed by most causes of death, however, they were detected in corpses killed by competition beginning from 0h postmortem time. Similarly, oleic acid was found in competition-killed corpses beginning from 0h postmortem, in hexaflumuron-killed corpses from 4h postmortem, in corpses killed by freezing, desiccation, fipronil and fungi from 8h postmortem, and from 16h in corpses killed by heating and imidacloprid.

For early death cues, cause of death showed a significant effect on the amount of 3-octanol ($F_{7,503} = 16.09$, $p < 0.001$), but no colony difference ($F_{2,503} = 2.31$, $p = 0.10$).

There was an interaction between colony and cause of death ($F_{14,503} = 3.45$, $p < 0.001$).

For 3-octanone abundance, there were significant effects in both cause of death ($F_{7,330} = 12.19, p < 0.001$) and colony ($F_{2,330} = 6.57, p < 0.01$), with interaction ($F_{14,330} = 4.21, p < 0.001$). For late death cues, cause of death showed a significant effect on the amount of indole ($F_{7,257} = 5.00, p < 0.001$) with colony effect ($F_{2,257} = 3.70, p = 0.03$) but no interaction between colony and cause of death ($F_{14,257} = 1.03, p = 0.42$). Cause of death ($F_{7,179} = 10.66, p < 0.001$) showed a significant effect on the amount of phenol, with an interaction between colony and cause of death ($F_{14,179} = 2.35, p < 0.01$). No colony effect was observed on the amount of phenol ($F_{2,179} = 2.77, p = 0.07$). Cause of death showed a significant effect on the amount of oleic acid ($F_{7,647} = 8.46, p < 0.001$) with no colony effect ($F_{2,647} = 0.16, p = 0.85$) or interaction between colony and cause of death ($F_{14,647} = 0.16, p = 0.99$).

2.4.2 Behavioral responses towards corpses with different causes of death at different postmortem times

According to the MCA analysis of eight causes of death, termites killed with fipronil and biotic natural causes, i.e. competition and fungi, were clearly separated from those killed by other causes of death (Figure 2.2). Death caused by fipronil was separated from other causes on the basis of dimension 1 (Dim1), which explained 63.5% of the total variance. Competition and fungi were separated from other causes on the basis of dimension 2 (Dim2), which explained 21.5% of the total variance. Specifically, corpses killed by fipronil were strongly associated with a number of contacts less than 25 (Chi-square = 116.93, $df = 21, p < 0.05$) and retrieval time longer than 40 min (Chi-square = 229.66, $df = 21, p < 0.05$). The separation of competition and fungi from other causes of death could

be explained by the way of disposition. Both causes of death were found to be significantly associated with burial (competition: Chi-square = 54.96, $df = 7$, $p < 0.05$; fungi: Chi-square = 21.14, $df = 7$, $p < 0.05$). In addition, desiccation was also suggested to be strongly associated with a number of contacts lower than 25 (Chi-square = 87.86, $df = 21$, $p < 0.05$). Other causes of death, including freezing, heat, imidacloprid, and hexaflumuron, were located close to cannibalism, retrieval time less than 40 min (categorized as <10, between 10 and 20, and between 20 and 40), and contact numbers over 25 (categorized as between 25 and 35, between 35 and 45, and >45) in the biplot, however, no significant association was suggested between any of these categories ($p > 0.05$).

Burial behavior was observed at different stages in decomposition among different causes of death (Figure 2.3A). Both postmortem time ($F_{7,191} = 545.96$, $p < 0.001$) and the interaction between postmortem time and cause of death ($F_{49,191} = 17.93$, $p < 0.001$) exhibited a significant effect on burial ratio. In corpses killed by biotic factors (competition and fungi), burial was observed at early postmortem stages (13.33% at 0h and 16.67% at 4h, respectively), and all corpses were buried after 32h postmortem time. In corpses killed by two short-term pesticides, fipronil and imidacloprid, burial was occasionally observed at 16h postmortem (3.33 and 16.67%, respectively). In corpses killed by freezing, desiccation, and hexaflumuron, burial was observed from 32h postmortem, whereas in those killed by heat, only corpses with 64h postmortem time were buried. There was no effect of colony ($F_{2,191} = 0.14$, $p > 0.05$) but a significant effect of cause of death ($F_{7,191} = 2.73$, $p = 0.01$) on burial ratio with no interaction ($F_{14,191} = 0.07$, $p > 0.05$). Corpses killed by competition and fungi were buried at a significantly higher

ratio than those killed by other causes of death at 4h (*Chi*-square = 18.86, DF = 7, $p = 0.009$), 8h (*Chi*-square = 18.73, DF = 7, $p = 0.009$), 16h (*Chi*-square = 19.95, DF = 7, $p = 0.006$), and 32h (*Chi*-square = 18.17, DF = 7, $p = 0.011$) postmortem time.

Corpses were retrieved from the testing chamber into the living chamber for cannibalism (Figure 2.3B). Fipronil-killed corpses took longer to retrieve than those killed by other abiotic factors across all postmortem times. Termites spent more time retrieving corpses killed by biotic factors than those killed by abiotic factors, except fipronil, at the 4h and 8h postmortem times. Cause of death showed a significant effect on retrieval time ($F_{7,1514} = 83.94$, $p < 0.001$), while colony ($F_{2,1514} = 2.19$, $p = 0.11$) and the interaction between colony and cause of death ($F_{14,1514} = 0.75$, $p = 0.73$) showed no effect. For corpses with 0h, 1h, and 2h postmortem time, it took termite workers significantly longer to retrieve fipronil-killed corpses than those killed by all other causes of death ($p < 0.001$). For corpses with 4h postmortem time, fipronil- and fungus-killed corpses took longer to retrieve than those killed by all other causes of death ($p < 0.001$). For corpses with 8h postmortem time, fipronil- along with fungus- and competition-killed corpses took workers longer to retrieve than those killed by other abiotic factors ($p < 0.001$). For corpses with 16h postmortem time, fipronil-killed corpses took longer to retrieve than those killed by all other abiotic factors and showed no significant difference with the retrieval times of fungus- and competition-killed corpses ($p < 0.001$). At 32h postmortem, fipronil-killed corpses showed significantly longer retrieval time than all other corpses except for hexaflumuron-killed ones ($p < 0.001$).

Termite workers contact corpses produced from different causes of death a different number of times for the first hour after corpse introduction across different

postmortem times (Figure 2.3C). Cause of death showed a significant effect on number of contacts ($F_{7,954} = 25.09$, $p < 0.001$), while colony showed no significant effect ($F_{2,954} = 0.63$, $p = 0.53$) and no interaction with cause of death ($F_{14,954} = 0.56$, $p = 0.90$). Fipronil- and desiccation-killed corpses induced the fewest contacts with living nestmates. Fipronil- and desiccation-killed corpses showed significantly lower numbers of contacts than those killed by other factors across all postmortem times except for 4h, 8h, and 64h ($p < 0.001$). Competition- and fungus-killed corpses showed significantly more contacts than those killed by other factors at 16 and 32h postmortem ($p < 0.001$). No significant difference in number of contacts was found across different causes of death at 64h postmortem time ($F_{7,119} = 1.59$, $p = 0.14$). The correlation coefficient calculation suggested a negligible correlation between retrieval times and number of contacts ($r(1513) = -0.24$, $p < 0.001$) (Hinkle et al., 2003).

2.5 Discussion

2.5.1 Death by biotic factors accelerated the release of late death cues in *R. flavipes*

Termite workers detect and moderate behavioral responses toward dead nestmates using a sophisticated chemical communication system (Sun et al., 2017). Specifically, after the death of an *R. flavipes* worker, two C8 volatiles, 3-octanol and 3-octanone were immediately released, reached a peak, and then gradually disappeared over the course of 16 hours across all modes of killing in this study. Fatty acids accumulated on the dead bodies during the subsequent decomposition process and two additional volatiles, phenol and indole, were also released. Chemical profiling across causes of death at different postmortem times revealed differences in the release of death-related chemicals at quantitative and qualitative levels (Figures 2.4 and 2.5). The release patterns indicated

that the selected biotic causes accelerate the release of late death volatiles and fatty acids. Fatty acids are known to be produced enzymatically from dead or wounded cells in the insect body via the breakdown of triglycerides (Blum, 1970; Akino and Yamaoka, 1996). Triglycerides and oleic acid were shown to decrease after protozoa in the gut of *R. flavipes* workers were (partially) eliminated by starvation (6 days), heat (35°C for 68 h), or high oxygen levels (1 h), showing that the oleic acid acted as a key source of energy during stress (Mauldin, 1977). The accelerated release of late death cues may be resulted from an accelerated decomposition process and/or the need for oleic acid as an energy source during stressful treatments.

Freezing-killed corpses showed a similar release of early and late death cues as in Sun et al., 2017. Heat- and desiccation-killed corpses showed similar release of oleic acid as freezing-killed ones. In our heat treatment, termites were kept at 44°C for only 25min, which might not provide enough time for termites to adjust the synthesis of oleic acid. When *R. flavipes* workers encounter inter- or intraspecific competitors, cues from the competitors can rapidly cause mortality, suggested because of decreased feeding and rest and increased termite activity (vibration and defensive behaviors) when under threat (Tian et al., 2017). The faster release of late death cues at early stages of decomposition in this study may be partially explained by the possibility that oleic acid from triglycerides may function as the energy source supporting higher activity with less food intake under competitive stress. In corpses killed by fungal infection, the accumulation of oleic acid indicated a high decomposition level at 16h postmortem, which was equivalent to the level observed at 64h in most corpses killed by abiotic factors. Similar to our results, in the fire ant, *S. invicta*, decomposition was also accelerated by fungal infection,

where oleic acid was found to be significantly higher in pupae killed by *M. anisopliae* after 24h postmortem (Qiu et al., 2015). The expression of genes related to hydrolytic enzymes is triggered in both the entomopathogenic fungus and insect host during infestation (Schrack and Vainstein, 2010), facilitating the hydrolyzation of triglycerides into oleic acid (Akino and Yamaoka, 1996). Thus, oleic acid may quickly accumulate in *R. flavipes* corpses as a result of fungal infestation.

2.5.2 Death by biotic factors accelerated the deployment of burial behavior in nestmate workers

In our study, death caused by biotic factors accelerated the employment of burial. Corpses recently killed (within 32h postmortem) by abiotic factors, such as freezing, heat, and desiccation, were retrieved for cannibalism and highly decayed corpses were buried. Specifically, in corpses killed by fungal infestation and competition, burial was employed at early stages of decomposition (4h for fungus, 0h for competition) and became dominant over cannibalism after 16h postmortem. Burial appears to be the most effective behavior for limiting disease transmission since it terminates all further interaction through physical isolation (Carter et al., 2007). The early employment of burial behavior in fungus- and competition-killed corpses might prevent subsequent risks associated with these causes of death. Termites intensify their responses to combat illness as the fungal infestation gets worse. After being infected by a congeneric fungus, *M. anisopliae*, *R. flavipes* workers increase allogrooming toward fungus-exposed individuals, transitioning to cannibalism after spore germination and consuming infected individuals alive (Davis et al., 2018). Our results then showed that when infection

became severe and fatal, cannibalism was still the primary strategy. As the corpses decomposed, burial was utilized. Similarly, when a congeneric termite, *R. virginicus*, was killed by *M. anisopliae*, fungus-killed corpses were buried, but not cannibalized as those killed with chloroform (Kramm et al., 1982). In the fire ant, *Solenopsis invicta*, fungal infection by *M. anisopliae* accelerated the removal of pupal corpses compared to unaffected ones potentially because of the accelerated release of oleic acid (Qiu et al., 2015). The use of burial in fungi-killed corpses began at 4 hours after death, although the considerable accumulation of oleic acid did not occur until 16 hours after death. This suggests that the early adoption of burial behavior may be partially explained by the increased accumulation of late death cues for corpses killed by fungi. For competition, earlier employment of burial would prevent potential encounters with competitors and subsequent injury and mortality. During the preparation of corpses killed by competition for this study, termites were observed to bury dead nestmates at < 24h postmortem. Similarly, encounters between two congeneric termite competitors, *C. formosanus* and *C. gestroi*, resulted in the blockage of tunnels leading to the site of conflict, where corpses were present (Li et al., 2010). The emergence of burial behavior coincided with the buildup of late death cues in the corpses killed by competition. Taken together, the faster accumulation of late death cues in this study might explain why burial behavior was adopted more quickly in corpses killed by biotic causes than in other causes of death.

Modes of killing were selected to simulate possible natural causes of mortality for *R. flavipes* workers. Freezing, heat and desiccation were used to generate corpses killed by naturally occurring abiotic factors. Subterranean termites are sensitive to changes in environmental factors, such as temperature and humidity (Sattar and Naeem, 2013). In

nature, subterranean termites can survive cold weather through physiological tolerance. Brief exposure to non-freezing temperatures is usually not lethal to termites. For *R. flavipes*, individuals can endure -2°C for at least 72 hours and the freezing temperature range is from -4 to -4.6°C (Clarke et al., 2013). Subterranean termites can adapt to seasonal temperature fluctuations by migrating deeper into the soil (Hu and Song, 2014). Freezing to death in *R. flavipes* colonies has been observed after a sudden drop in temperature (personal observation). In a previous study, corpses of *R. flavipes* workers killed with -20°C in 15 min elicit the same cannibalizing response in less than 24 hours postmortem time (Sun et al., 2013). Freezing-killed corpses in this study were generated using -80°C with 1 min to simulate a sudden frozen death with relatively accurate time 0 of the postmortem time at the cost of ecological relevance. Further research is necessary to determine whether there are any physiological and/or chemical variations between corpses who died at various freezing temperatures. *R. flavipes* has been suggested to be more tolerant of cold than warm (Hu and Appel, 2004). Temperature in heat treatment was chosen based on the critical thermal maximum of *R. flavipes* with a range from 43.5 to 44.9°C (Hu and Appel, 2004). An extreme temperature increase can cause mortality in *R. flavipes* nest. Termites are also highly susceptible to desiccation. In a previous study, 0% relative humidity (RH) caused 50% mortality in around 24h in an *R. flavipes* colony with 100 individuals (Janowiecki and Vargo, 2021). Both group size and relative humidity can affect the rate of death due to desiccation. In our preliminary study, the majority of 50 *R. flavipes* workers kept in the same dish may still be alive after 24 hours at 30% RH, but when only ten were contained in the same dish, all treated termites were dead. Termite workers frequently die when defending the colony against external biotic

agents. Disease is one of the most common causes of death for termites (Sun et al., 2018). Termite colonies exhibit a group-living style in cryptic nests with limited genetic variation, making them suitable targets for pathogens (Cremer et al., 2017). However, epizootic rarely happens in termite colonies (Keller and Zimmermann, 1989). The entomopathogenic fungi, *Metarhizium* sp., are widely present in nature and possess a high level of virulence against *R. flavipes* (Lu and St leger, 2016; Vivekanandhan et al., 2020). Initiation of an epizootic using *Metarhizium* sp. as commercial biological agents in termites has been tried for many years but remains unsuccessful (Chouvenc et al., 2011). As shown in our results, proper behavioral responses to dead conspecifics killed by fungal infestation could reduce the possibility of epizootic and partially account for the failure of using *Metarhizium* sp. in biological control against termites (Chouvenc et al., 2011). Termite workers can also die in their defense against predators and competitors. Field colonies of *R. flavipes* can locate close to each other with overlaps (Janowiecki and Vargo, 2021). Corpses generated from competition can cause tunnel blockages to prevent further interactions between nestmates and inter- or intraspecific competitors (Li et al., 2010), which has been suggested as an important factor in regulating territories between colonies in subterranean termites (Jones and Trosset, 1991). Contactless competition from intraspecific competitors could elicit mortality in *R. flavipes* (Tian et al., 2015). In this study, two groups of *R. flavipes* workers from different colonies were put in the same set-up as Tian et al., 2015 to simulate corpses from indirect intraspecific competition. The accelerated burial behavior toward corpses generated from intraspecific competition can not only prevent future encounters with intraspecific competitors but also contribute to the regulation of colonial boundaries (Li et al., 2010). The mechanism of mortality from

intraspecific contactless competition is still unclear. Meanwhile, corpses generated from other competitive/predatory conditions require future investigations.

The capability to identify dead conspecifics and respond to them accordingly is integral to group-living animals (Sun et al., 2013; Sun et al., 2018; Gonçalves and Biro, 2018).

Such death-related behavioral plasticity has been reported in eusocial insects as differential responses to corpses based on the nature of the corpse, including decomposition status (Diez et al., 2013; Sun et al., 2017), corpse origin (Sun et al., 2013; Qiu et al., 2020), caste/developmental stage (Munday et al., 2018; Shi et al., 2021) and cause of death (Qiu et al., 2015; Noeh et al., 2012). Different causes of death may be associated with different types of risks. Conspecific death can pose a direct risk to the living, for example, through hazardous byproducts of decomposition (Til et al., 1997), contact or stomach poisoning pesticides (Rust and Saran, 2006), pathogens (De Hoog, 1996, Büttner and Bonas, 2003, Parvez and Parveen, 2017), protozoans, and parasitic worms (Hart, 1990; Fayer et al., 2004). Death of a conspecific individual could also represent indirect risks, for example, corpses generated from aggressive encounters with predators or competitors might suggest risks of potential conflict with nearby predators or competitors (Li et al., 2012; Iglesias et al., 2012); corpses killed by freezing, heat, or desiccation might indicate the potential risk of extreme climate/weather change (Mellanby, 1939; Ratnayake et al., 2019). In nature, termites have evolved to deal with drastic climate changes through physiological thermal tolerance (Hu and Appel, 2004; Clarke et al., 2013), behavioral responses (moving downward in the ground) (Hu and Song, 2014), and maintenance of heat and humidity within the nest (Korb, 2003; Ptáček et al., 2013).

2.5.3 Death caused by pesticides and naturally occurring abiotic factors cannot be distinguished by nestmate workers

The results of this study show that in *R. flavipes*, selected pesticides (fipronil, imidacloprid and hexaflumuron) did not impact the disposition of conspecific corpses. Freshly killed corpses (less than 32h postmortem) were retrieved for cannibalism and highly decayed corpses (64h postmortem) were buried on site, comparable to corpses killed by abiotic factors (freezing, heat, and desiccation). Similarly, laboratory studies showed that soil treated with fipronil and imidacloprid didn't repel *R. flavipes* and could effectively prevent them from penetrating because of the increase in mortality (Remmen and Su, 2005; Gahlhoff and Koehler, 2001). Both imidacloprid and fipronil exhibit neurotoxicity to termites. Fipronil (phenyl pyrazole) kills termites by obstructing GABA-regulated chloride channels and eliciting hyper-excitation, while imidacloprid (neonicotinoid) paralyzes and kills by blocking nicotinic acetylcholine receptors (Narahashi et al., 2007; Zhang et al., 2000). When used as active ingredients in soil treatments, fipronil and imidacloprid show a half-life degradation from 93 to 226 days and from 26.5 to 229 days, respectively (Tingle et al., 2003; Fossen, 2006). As used in registered commercial products, 0.0625% fipronil and 0.4% imidacloprid application in concrete slab tests remained complete protection for 9 and 4 years, respectively (Wagner, et al., 2002). The long duration and lack of repellency ensured termites were not deterred by the treated soil and contacted lethal doses of chemicals. Both of them can be transferred among individuals within colonies (Shelton et al., 2003; Saran and Rust, 2007; Haagsma and Rust, 2007) and induce colony elimination (Parman and Vargo,

2010; Vargo and Parman, 2012). Imidacloprid has also been suggested to enhance the susceptibility of *R. flavipes* to fungi and nematode infestation. Such synergistic effects when using imidacloprid and entomopathogens together may facilitate colony effect of both methods in subterranean termite treatments (Ramakrishnan et al., 1999; Manzoor et al., 2012). The chitin synthesis inhibitor hexaflumuron shows population suppression in *C. formosanus* and *R. flavipes* with a wide effective range of concentrations (Su, 1994). Hexaflumuron has been demonstrated to suppress ecdysis in *Reticulitermes* and *Coptotermes* termites (Su and Scheffrahn, 1993 and 1998; Su, 1994). Similar to Su and Scheffrahn, 1993, affected termites exhibited a "jackknife" stance during the next molting, bending their bodies at the intersection of the thorax and abdomen. Hexaflumuron's effects resulted in partial separation of newly generated cuticles and exuviae. Cannibalism might occur as a result of injury from other nestmates who assisted with the molting (Su and Scheffrahn, 1993). Our findings also supported the cannibalism of newly dead nestmates killed by hexaflumuron. Corpses killed by exposure to these chemicals are still retrieved by nestmate workers before they become highly decayed and are collectively cannibalized, suggesting that undertaking behavior may promote pesticide transference and efficiency within colonies.

The main difference occurs in the process of disposition of the corpses. Fipronil-killed corpses are physically contacted fewer times by nestmates and take longer to retrieve, indicating a delay in undertaking recently killed corpses. Our results didn't support a correlation between the retrieval times and contact numbers, suggesting the longer retrieval time was not necessarily resulted from the lower contact number. Horizontal transfer of fipronil can immobilize healthy nestmates, which may assist in

explaining the delay in corpse retrieval. The immobilization and delay of undertaking freshly fipronil-killed corpses might result in the accumulation of corpses in treated areas. Insects can reduce the efficiency of a pesticide to insecticides by changing their behavior (Onstad and Knolhoff, 2014). For instance, the German cockroach, *Blattella germanica*, avoids feeding on sugar baits containing a toxicant due to a change in the glucose receptor (Hostetler and Brenner, 1994; Wang et al., 2004). In termites, behavioral resistance has also been observed for fipronil treatment. In *C. formosanus*, the accumulation of dead and decayed corpses in areas treated with fipronil can prevent other nestmates from entering these areas and reduces the efficacy of the termiticide (Su, 2005). In *C. formosanus*, with ideal concentrations, fipronil treatment is less effective over 5m away from the treated area (Su, 2005), whereas in *R. flavipes*, this distance has been suggested to be over 10m (Parman and Vargo, 2008). In our study, fipronil-killed corpses were dragged back toward the colony (away from the treated site) and subsequently cannibalized, leading to the immobilization of nestmates, suggesting an explanation for this extension of the effective area of fipronil. By using C14-labeled fipronil, horizontal transfer was suggested through body contact, not trophallaxis (Saran and Rust, 2007), with nearly 50% of fipronil transferred from exposed termites to other nestmates (Bagnères et al., 2009). This dilution during horizontal transfer would partially explain the recorded effective distance. Our study suggests that this resistance to fipronil in subterranean termites is more behavioral than physiological.

2.6 Conclusions

Death in termites can be attributed to various environmental factors along with termite control practices, which in turn pose different risks to the colony. Death-related chemicals

differ according to their mode of action and change over time. In this study, we integrated ethology with chemical ecology approaches in the eastern subterranean termite, *R. flavipes*, to test the hypothesis that termites exhibit different undertaking responses to nestmate corpses generated from different causes of death as corpses age. With all results integrated, we found that termite workers are unable to differentiate nestmates killed by pesticides from those killed by naturally occurring abiotic factors, whereas they can detect the accelerated release of late death cues in corpses killed by biotic factors and employ burial promptly. The observed differences in behavioral responses can be explained by the quantitative and qualitative differences in postmortem chemical profiles across different causes of death. The differential behavioral responses suggest functions in behavioral resistance of termites against disease, competitors, and potentially pesticides. This study advances our knowledge of corpse management in social insects and provides insight into the underlying mechanism of behavioral resistance to chemical controls in a destructive subterranean termite.

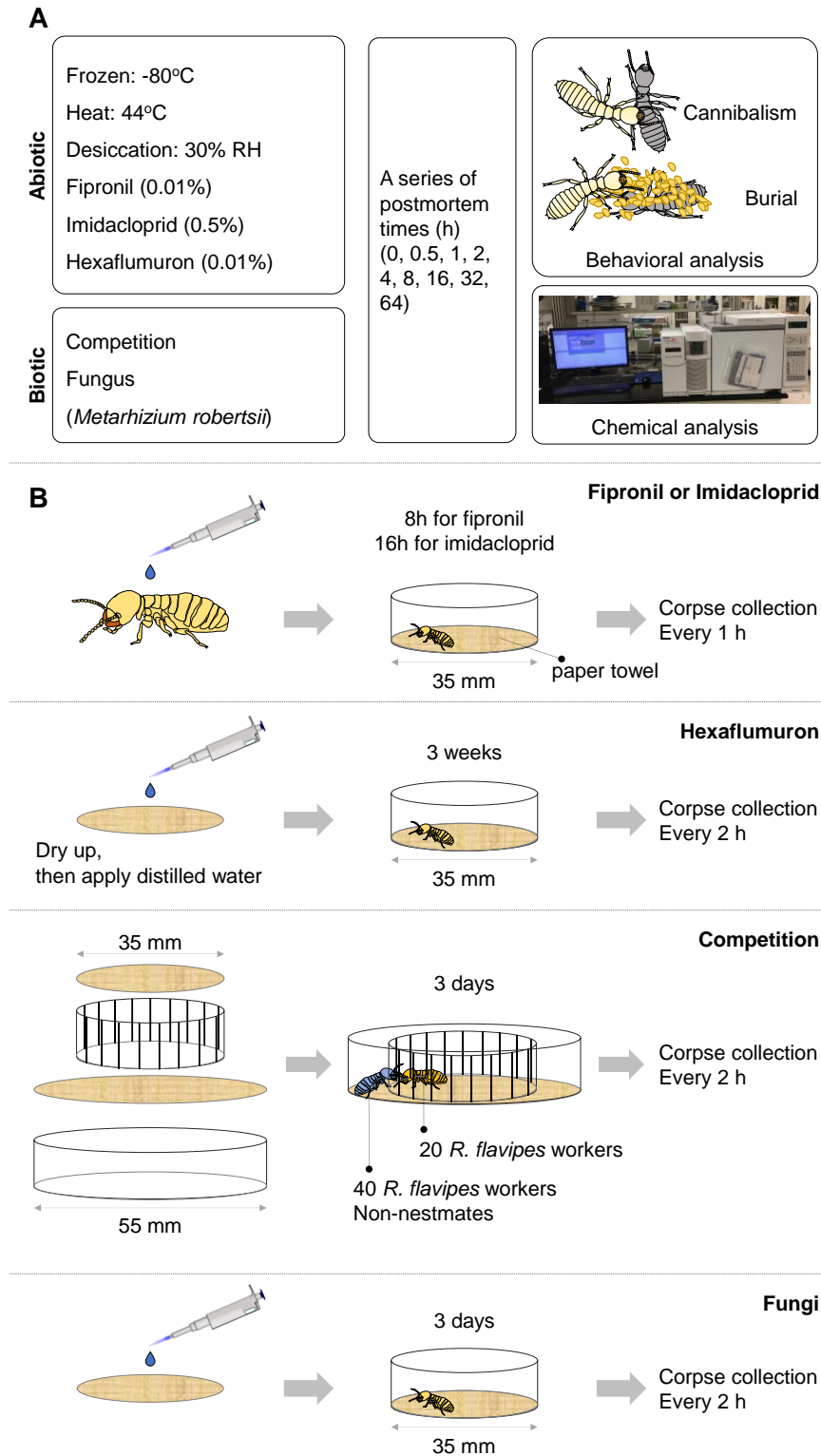


Figure 2.1 Experimental design and workflow.

Workflow in this study has been illustrated based on main steps. **(A)** *R. flavipes* workers were treated with different factors to generate different types of corpses, including freezing, heat, desiccation, synthetic pesticides (fipronil, imidacloprid, hexaflumuron) as abiotic factors and contactless competition between non-nestmate conspecifics and fungal infection as biotic factors. All collected corpses were stored in the same conditions for a series of postmortem times. Corpses were then either introduced to a behavioral assay or extracted as chemical samples for GC-MS analysis. **(B)** Main steps for fipronil, imidacloprid, hexaflumuron, competition, and fungus treatments.

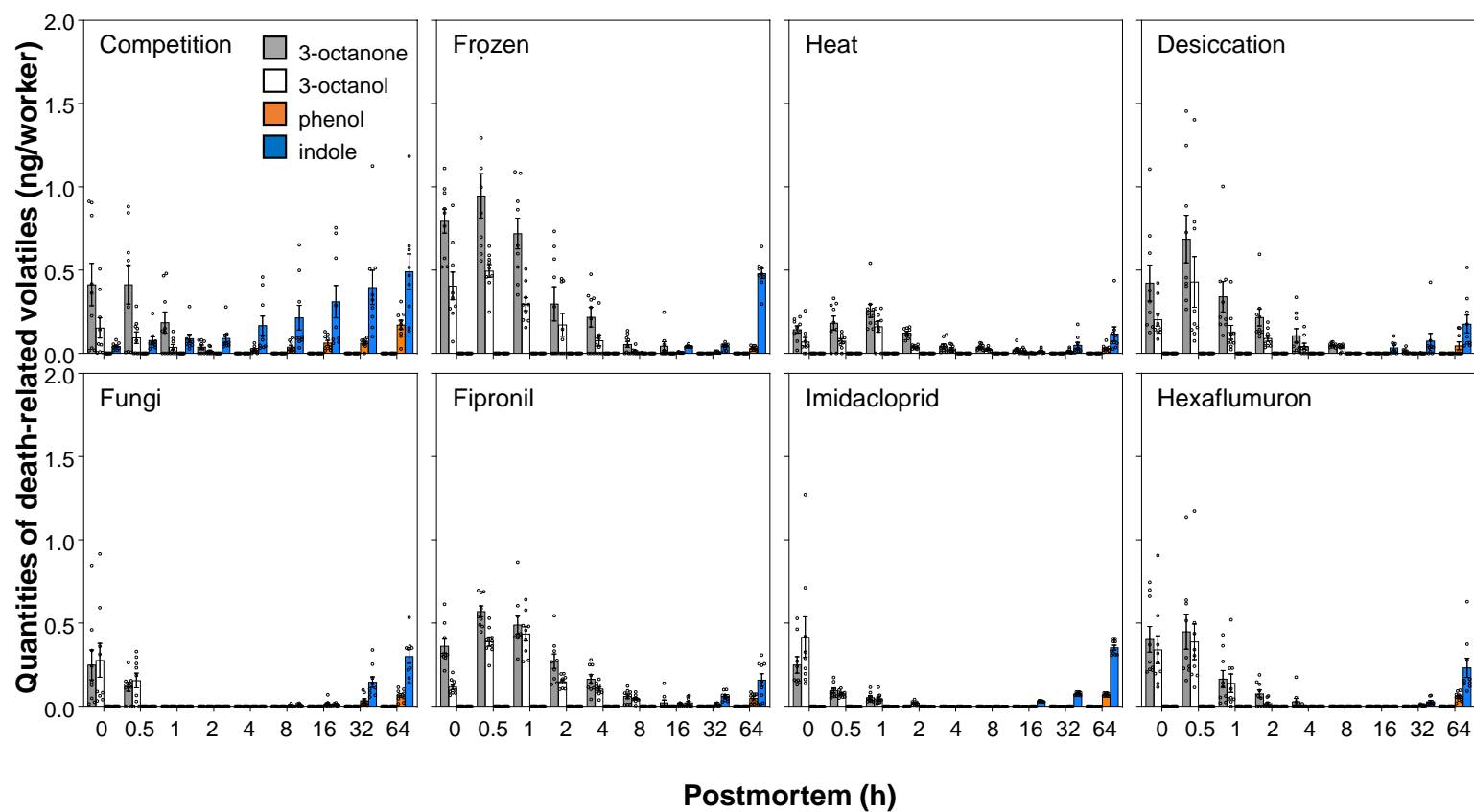


Figure 2.2 Release patterns of death-related volatiles across different causes of death in *R. flavipes*.

Temporal changes of quantities of early and late death volatiles were shown under different causes of death across different postmortem times (Mean \pm SEM). Distributions of samples were represented by unfilled circles within each group. Volatiles

were colored with different colors (3-octanone: grey; 3-octanol white; phenol: orange; indole: blue). Data were pooled from 3 colonies with 3 replications for each colony.

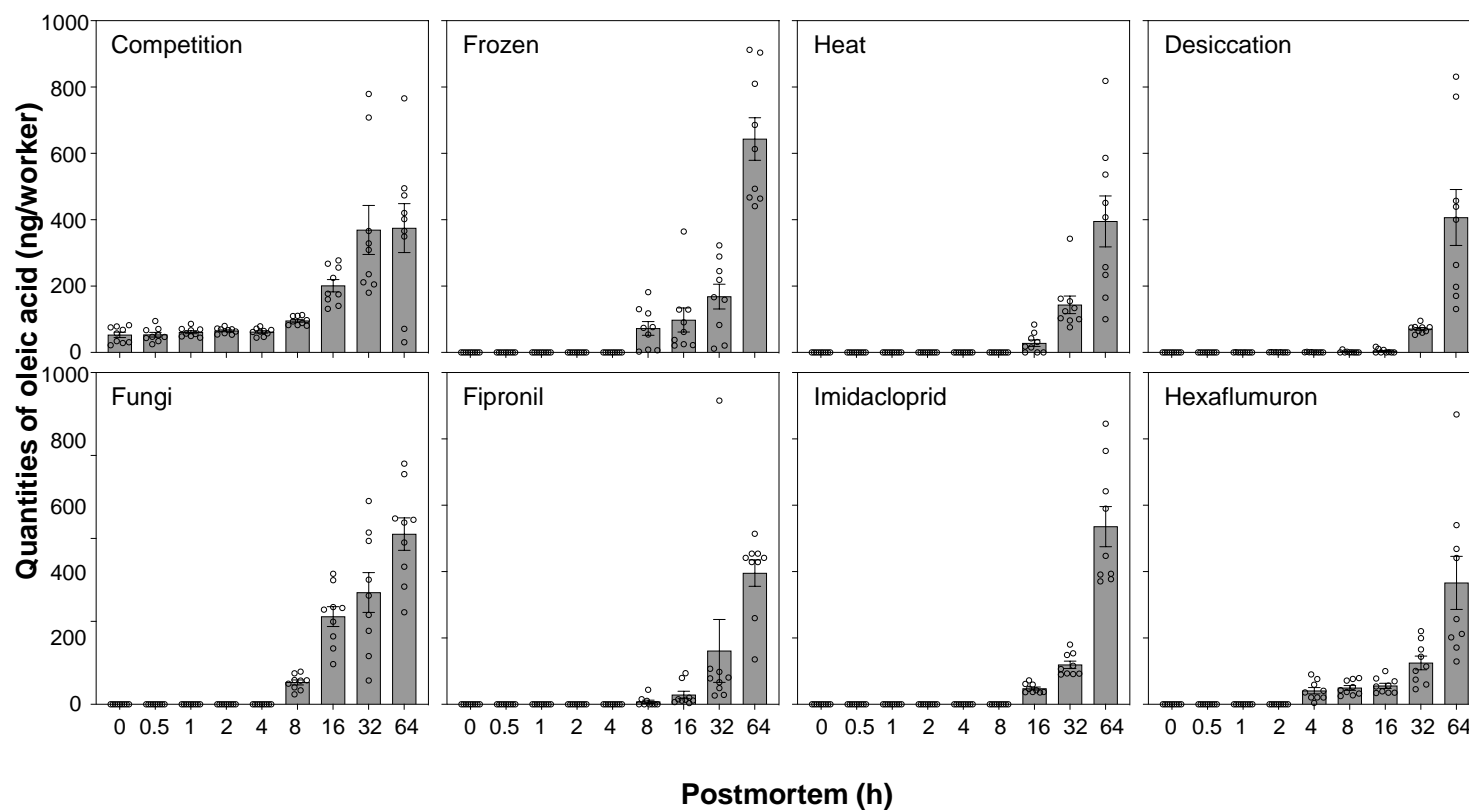


Figure 2.3 Dynamic of detected amount of oleic acid on worker corpses across different causes of death.

The panel shows accumulation of oleic acid along with decomposition among different causes of death. Amount of oleic acid was quantified using hexane extraction (Mean + SEM with sample distribution). Samples in each group were represented by black dots. Data were pooled from 3 colonies with 3 replications for each colony.

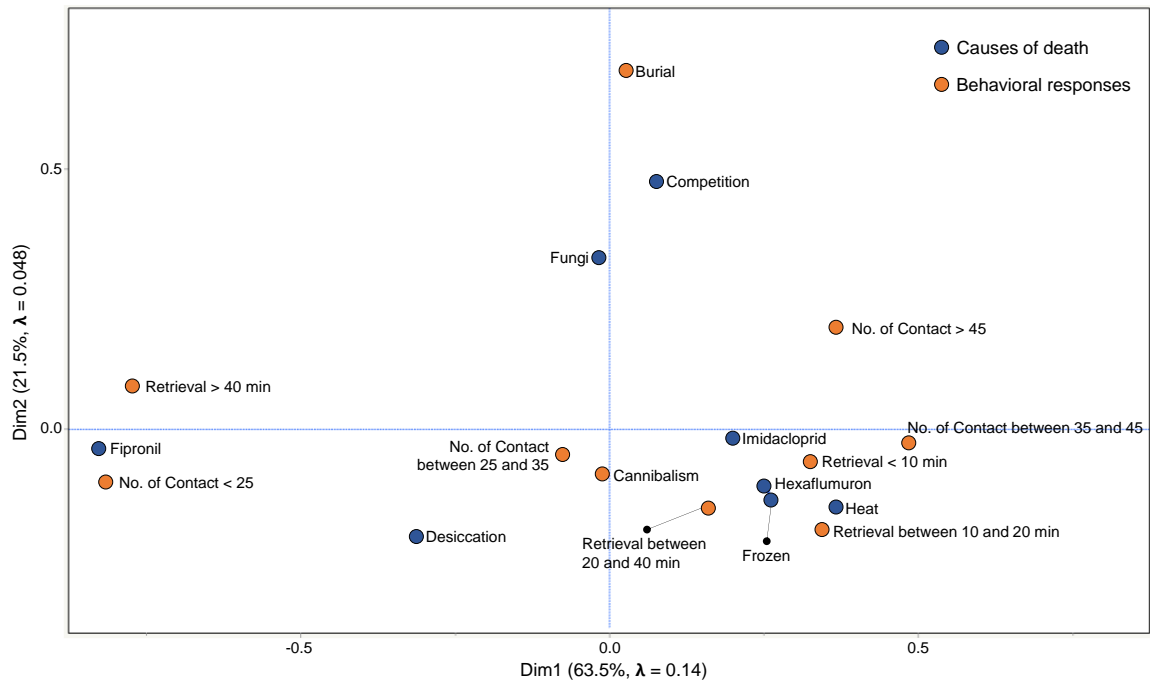


Figure 2.4 Multiple correspondence analysis: causes of death against death-related behaviors.

Biplot represents results from the multiple correspondence analysis with dimensions 1 (Dim1) and 2 (Dim2). Causes of death were colored in blue and behavioral responses in orange. Specifically, blue circles represented eight different causes of death. Orange circles showed three death-related behavioral characteristics, disposition (cannibalism and burial), number of contacts between living workers and the corpse within 1 h after the introduction of the corpse (4 categories: No. of contact <25, between 25 and 35, between 35 and 45, > 45), and retrieval time (4 categories: Retrieval < 10, between 10 and 20, between 20 and 40, > 40 min).

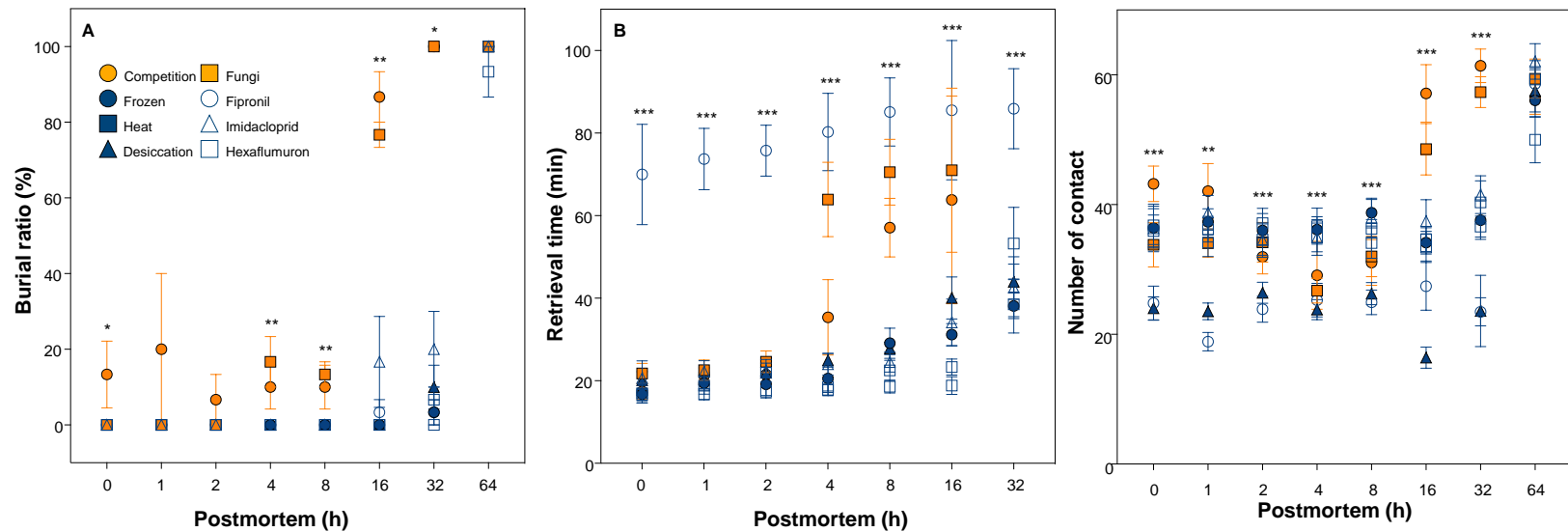


Figure 2.5 Behavioral responses toward corpses with different causes of death.

Figures show temporal change of behavioral responses of termite workers toward dead conspecifics with different causes of death: A. burial ratio in all 10 replicates (Mean \pm SEM); B. retrieval time by workers (min) (Mean \pm SEM); C. counts of contact toward newly introduced corpses (Mean \pm SEM). X-axis indicates different postmortem time points (h). Biotic causes of death were colored yellow, whereas abiotic causes were colored blue. Different causes of death within each category were marked as different symbols, respectively. Significance across different causes of death at each time point was labeled with asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

CHAPTER 3. MANAGING CORPSES FROM DIFFERENT CASTES IN THE EASTERN SUBTERRANEAN TERMITE, *RETICULITERMES FLAVIPES* (BLATTODEA: RHINOTERMITIDAE)

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3.1 Abstract

Corpse management is essential for social animals to maintain colony health. In the eastern subterranean termite, *Reticulitermes flavipes*, workers carry out undertaking behaviors to mitigate the risks associated with the dead. In this study, we hypothesized that termites would respond differently to the corpses from different castes based on their postmortem chemical signatures.

To test this hypothesis, we 1) documented the behavioral responses of the workers toward corpses from different castes, and 2) profile the chemical signatures of these corpses. Corpses from all castes were retrieved inside the nests and cannibalized when they were decomposed <64 h, regardless of the presence or absence of the cues that we refer to as early death cues (3-octanol and 3-octanone). However, after 64h, all corpses except for soldiers were buried on site by *R. flavipes* workers. The late death cues (oleic acid) were cumulative over time among castes but accumulated more slowly and at lower levels in soldiers. The differential release of 3-octanol and 3-octanone between

workers/soldiers and nymphs could be explained by either qualitative or quantitative differences in signaling the death between imaginal and neuter developmental pathways.

In summary, the efficient and selective recognition of the dead and the fine-tuning of subsequent undertaking responses observed in *R. flavipes* are aspects of corpse management, which can minimize the potential risks associated with different castes and maximize the colony fitness.

3.2 Introduction

Death is frequently encountered by animals, and represents a potential pathogenic risk (Liu et al. 2019), which typically induces avoidance behaviors by living conspecifics in both vertebrates (Stroud et al. 2014, Pinel et al. 1981, Carr et al. 1981, Prounis and Shields 2013, Montoya et al. 2004) and invertebrates (Yao et al. 2009, Candia-Zulbarán et al. 2015, Aksenov et al. 2017, Kierat et al. 2017). This is particularly true in eusocial insects, in which corpse management is essential to coping with the risks associated with the dead due to their enclosed nesting structures (Wilson et al. 1958, Shelton 2014, Sun and Zhou 2013) and high population densities (Su et al. 1993, Hölldobler and Wilson 2009). Necrophoric behavior, i.e., removal of dead colony members, has been observed in Hymenoptera (Wilson et al. 1958, Visscher 1983, McAfee et al. 2018, López-Riquelme et al. 2013). Whereas in termites, cannibalism is primarily conducted to recycle nutrients, such as nitrogen (Sun et al. 2018, Chouvenc 2020, Sun et al. 2020), while defense against pathogens, such as burial behavior, is a secondary benefit (Nalepa 1994). When there is battle or competition, decayed corpses would result from walling-off of intrusion points, where termites were killed, and subsequently rediscovered only while the tunnels are reopened (Li et al 2010, Lima and Costa-Leonardo 2012, Bernard et al

2017). As the corpses accumulate and continue to decompose, cannibalism becomes inefficient, and burial behavior would then follow (Strack 1998, Myles 2002, Chouvenc and Su 2012, Sun et al 2018, Davis et al. 2018).

Recognition of death is the critical first step for social animals to elicit undertaking behavior. Death recognition in eusocial insects primarily relies on chemical cues. The involvement of non-olfactory cues, such as tactile, auditory, thermal, or visual cues, is largely unknown, with the exception of a synergistic effect of tactile cues and a chemical death cue, oleic acid, on burial behavior in a subterranean termite, *Reticulitermes virginicus* (Ulyshen and Shelton 2012). Currently, there are two major hypotheses underlying chemical cue-based death recognition: 1) the increased death cue hypothesis, suggesting that the accumulation of death-related chemicals elicits undertaking behavior (Wilson et al. 1958, Sun and Zhou 2013, Sun et al. 2018), and 2) the diminished vital sign hypothesis, proposing that the decrease of chemical cues associated with life immediately upon death mediates undertaking behavior (Choe et al. 2009). Since the discovery that the postmortem release of fatty acids stimulates corpse removal in ants (Wilson et al. 1958), fatty acid death cues have not only been documented in social insects, but also widely reported to be repellents across other arthropod taxa (Haskins and Haskins 1974, Howard and Tschinkel 1976, Yao et al. 2009, Chouvenc et al. 2012, Ulyshen and Shelton 2012, Diez et al. 2013, McAfee et al. 2018). In comparison to the well-documented chemical releaser of undertaking behaviors in fatty acids, the diminished vital sign has only been identified in one ant species, the Argentine ant, *Linepithema humile* (Choe et al. 2009). Recently, a third hypothesis has been proposed in the eastern subterranean termite, *Reticulitermes flavipes*, in which the

interplays between the early and late death cues trigger a behavioral switch from cannibalism to burial in *R. flavipes* to balance pathogenic risks and nutritional rewards associated with corpses (Sun et al. 2017). Specifically, the combination of fatty acids, phenol and indole are considered as late death cues due to their gradual accumulation after death (Chouvenc et al. 2012; Sun et al. 2013), whereas, 3-octanone and 3-octanol, which are released immediately upon death, peak at 0.5 h, diminish at 8h postmortem, and elicit the retrieval of conspecific corpses from the testing arena to the holding chamber within 0.5 h after death (Sun et al. 2013), are categorized as the early death cues (Sun et al. 2017).

In a natural termite colony, how and how often could the colony members encounter dead conspecifics from different castes? In eusocial animals, individuals follow different developmental trajectories and specialize in certain roles (Hölldobler and Wilson 2009). The developmental pathway in *Reticulitermes* can be divided into an imaginal (winged) and a neuter (wingless) lines (Lainé and Wright 2003). After initial embryonic and larval stages, *R. flavipes* follow either the imaginal line to develop into nymphs or the neuter line to molt into workers. Both primary reproductives, i.e., kings and queens, and secondary reproductives, nymphoids, are derived from nymphs. When undertakers encounter a dead termite, it is most likely a worker, not only because of their sheer dominance in numbers (Howard and Haverty 1981), but also risks associated with their allocated tasks (Bignell et al. 2010). Howard and Haverty (1981) surveyed the caste composition of *R. flavipes* in infested wood logs, in which workers were the most abundant caste (84%), followed by larvae (8.7%), nymphs (5%), soldiers (2.1%), pre-soldiers (0.1%) and neotenic reproductive (0.1%). Castes involved in risky tasks could be

the most frequent source of day-to-day mortality. For example, workers handle a wide range of the tasks, including foraging, feeding, brood care and nest maintenance/colony hygiene (Bignell et al. 2010), nymphs can develop into reproductives (Noirot and Pasteels 1987), and soldiers serve a defensive role to fend off competitors or predators (Yanagihara et al. 2018, Mitaka and Matsuura 2020). Given that foragers, i.e., older workers, are exposed to the outside world beyond the protective nesting structures (Sakagami and Fukuda 1968; Darlington, 1991; Schmid-Hempel and Schmid-Hempel 1993; Du et al. 2016), and soldiers involve direct physical combat with competitors or predators, worker and soldier are the most risk-taking castes.

In this study, our goal is to study how termites cope with the corpses derived from these different castes. Our overarching hypothesis is that *R. flavipes* workers would respond differently to the corpses from different castes based on their postmortem chemical signatures. To test this hypothesis, we carried out the following objectives: 1) documented the behavioral responses toward corpses from workers, soldiers, and two types of immature reproductives (nymphs with short or long wing buds) across a range of postmortem times; and 2) profiled the chemical signatures of these corpses.

3.3 Materials and methods

3.3.1 *Reticulitermes flavipes* colony maintenance

Reticulitermes flavipes colonies were collected between May and October 2018. The colonies were collected from two separated sites at Red River Gorge within the Daniel Boone National Forest (R-I: 37° 47' 38" N 83° 35' 55" W and R-II: 37° 47' 20" N 83° 35' 42" W) and one site at the Arboretum State Botanical Garden of Kentucky, which is located on the campus of the University of Kentucky (A: 38° 00' 54" N 84° 30' 28" W).

Termite traps were made from cardboard rolls (diameter = 8.5cm, height = 15.2 cm, wrapped by a rubber band) and placed under rotten fallen wood trunks. All traps were at least 200m away from each other (Figure 3.1A). Samples were collected every week, and traps were replaced after each collection. Termites were extracted from collected cardboard rolls, then placed in a Petri dish (diameter = 14.5 cm, height = 2.0 cm) with dampened unbleached paper towels as a food source. where they were held for two weeks. After that, termites were transferred to a cuboid plastic shoebox and provisioned with moistened wood mulch and pine woodblocks. Termites collected from the same sites (the same rotten trunk) continuously throughout the season were considered as the same colony and kept in the same shoebox. *Reticulitermes flavipes* colonies were maintained in an incubator with L:D = 0:24 at 27 ± 1 °C, and 89-99% RH. Before behavioral and chemical analysis, castes were identified based on morphological traits following Lainé and Wright (2003). Specifically, workers are wingless individuals lacking compound eyes with a pronotum that is trapezoidal in shape. Soldiers are individuals with elongated defensive mandibles, while their thoraxes and abdomens are similar to those of workers. Nymphs can be subdivided into those with short wing buds (SWBN), and those with long wing buds (LWBN) (Figure 3.1C). In the laboratory, field-collected colonies were placed in a black PVC bucket for caste identification and separation (Figure 3.1A).

3.3.2 Behavioral responses toward corpses from different castes

The undertaking behavioral response towards termite corpses from workers, soldiers, short wing-bud nymph (SWBN) and long wing-bud nymph (LWBN) was tested in 3 *R*.

flavipes colonies with 8 replications for each colony (Figure 3.1B). A Petri dish (diameter = 5.5cm, height = 1.5cm), the living chamber, was connected to a smaller dish (diameter = 3.5cm, height = 1.5cm), the testing arena, using a plastic tube (inner diameter = 0.7cm, length = 3.5cm). An entry port (diameter = 0.5cm) was drilled onto the lid of the testing arena to introduce the corpses. Unbleached paper towel discs were lined at the bottom of each Petri dish and treated with 200 μ L of distilled water, to serve as the food source. One gram of moistened sand (15% water by weight) was put into the living chamber as burial materials. A total of 29 workers and 1 soldier were placed in the living chamber. Termites were acclimated to the dishes for three days before the assay. *Reticulitermes flavipes* workers and soldiers were killed by freezing in a -80 °C freezer for 1 min, while nymphs required 3 min. The corpses were thawed at room temperature in covered Petri dishes for 5 minutes, then maintained in the same conditions as the previous colony rearing condition for a series of postmortem times (0, 1, 16, 32 and 64 h) before the behavioral assay. The corpse was placed in the testing arena at the start of the behavioral assay. Only one corpse from the same colony was introduced each time. Undertaking behavior was recorded with a video camcorder (Canon VIXIA HF G10, Canon Inc., Tokyo, Japan) for 24 hours. Several different behavioral parameters were recorded. The corpse was documented as retrieved if it was moved to the living chamber. The duration from the introduction of the corpse to first contact by workers was documented as the first detection time. The duration from the introduction of the corpse to its retrieval to the living chamber was recorded as the retrieval time. The duration of retrieval, however, was defined as the difference between the first detection time and the retrieval time.

Burial (depositing sand on the corpse) and wall-off (using sand to block the entrance to the testing arena) behavior were documented if observed in the 64h postmortem corpses.

3.3.3 Chemical profile of corpses from different castes

To profile the chemical signatures of corpses from different castes, we used an Agilent Technologies 6890N gas chromatograph (GC) in splitless mode with a DB-5 capillary column (30 m \times 0.25 mm \times 0.25 μ m; Agilent Technologies, Santa Clara, CA, USA). Helium was used as the carrier gas (1.0 mL/min). The starting temperature of the column, 40°C, was held for 2 min before being raised to 320°C at a rate of 10°C/min. The temperature of the injection port was 280°C. Mass spectra were obtained with an Agilent Technologies 5975 mass spectrometer (MS). The National Institute of Standards and Technology/National Institutes of Health/Environmental Protection Agency Mass Spectral Library was used as the basis for compound identification along with retention time from gas chromatography.

Termite corpses of workers, soldiers, SWBN and LWBN with a series of postmortem time (0, 0.5, 1, 2, 4, 6, 8, 16, 32, 64h) were collected as samples. A total of 15 individuals were put into a 2 mL glass vial sealed with an aluminum crimp cap with a pre-fitted septum (Thermo Fisher Scientific, Waltham, MA, USA). Volatiles were collected from the headspace of corpses with a 100 μ m polydimethylsiloxane solid-phase microextraction (SPME) fiber (Sigma-Aldrich, St. Louis, MO, USA). The needle of the fiber holder was injected into the vial and the fiber was extended inside without contacting the termite samples, and left there for 15 minutes for volatile collection. After

this step, the fiber was retracted and then manually injected into the inlet port of the GC for 1 min.

Quantification of compounds previously detected via SPME was conducted through hexane extraction of corpses. Discriminating ions and retention times were used to quantify the volatiles since the peak area was small and sometimes buried in contaminants. The peak area of the 99 and 101 ions was identified as indicators of 3-octanone and 3-octanol, respectively. Fifteen corpses were used for each sample and were first submerged in 200 μ L hexane (glass-distilled, Thermo Fisher Scientific, with 10 ng/ μ L n-nonadecane as internal standard) for 10 minutes. A total of 50 μ L of the solution was transferred to a conical bottom glass vial sealed with the same aluminum cap. Two μ L of solution was injected into the GC for analysis. A total of 8 replications for each type of corpse were generated from three *R. flavipes* colonies.

Though termites release a blend of fatty acids with phenol and indole as late death cues, burial behavior can be triggered by oleic acid alone (Sun et al. 2017). As such, we used the quantity of oleic acid from the corpses as an indicator of fatty acid abundance in late death cue analysis. Samples of 10 individuals were extracted using 300 μ L of glass distilled hexane with 10 ng/ μ L n-nonadecane as the internal standard for 10min. 200 mL of BF₃-methanol (10%, w/w) was added to the solution for 10min at 60 °C. The methyl ester derivative of the fatty acid was identified and quantified by GC-MS. Termite corpses from workers, soldiers, SWBNs and LWBNs at 16h, 32h, and 64h postmortem were subjected to analysis.

3.3.4 Data analysis

Statistical analysis was performed using SAS software, Version 9.4 (SAS Institute, Cary, NC, USA). Before the analysis of variance (ANOVA), the first detection time was log-transformed ($\log(x+1)$), the quantities of 3-octanone and 3-octanol were log-transformed as $\log(x)$ to fit the assumptions of parametric tests. The first detection time and the duration of retrieval were analyzed by two-way ANOVA with postmortem time, caste, and the interaction between the two as factors, while significance across different castes was analyzed using Tukey's HSD all-pair wise comparisons test. Two-way ANOVA was also used to compare the release of death cues with postmortem time, caste, and the interaction between them as factors. At each postmortem time point, we conducted one-way ANOVA to compare the average quantity of death cues across different castes. After conducting ANOVA, the normality of residuals was assessed visually by the normal quantile plots.

3.4 Results

3.4.1 Behavioral responses toward corpses from different castes

Corpses of all castes with less than 64h postmortem time were retrieved from the testing arena to the living chamber and then cannibalized by workers. After a termite worker detected the corpse, they began to drag the corpse using their mandibles and eventually brought the corpse back to the living chamber (Figure 3.2A). There was a significant effect of caste on the first detection time (ANOVA: $F(3, 382) = 7.48, p < 0.001$). It took workers a significantly longer time to locate LWBN corpses than worker and soldier corpses (Tukey's HSD all-pair-wise comparisons test; LWBN vs worker/soldier: $p < 0.001$; SWBN vs worker/soldier/LWBN and worker vs soldier: $p > 0.05$) (Figure 3.2B).

There was no effect of postmortem time and the interaction between postmortem time and caste on the first detection time (ANOVA; postmortem time: $F(3,382) = 1.75, p = 0.16$; interaction: $F(9,382) = 1.30, p = 0.24$).

There was a significant effect of caste of corpse on duration of retrieval (ANOVA: $F(3, 372) = 4.06, p < 0.01$). It took a significantly longer time for workers to retrieve SWBN corpses than those of the other three castes (Tukey's HSD all-pair-wise comparisons test; for SWBN vs worker/soldier: $p < 0.05$; for SWBN vs worker/soldier/LWBN and worker vs soldier: $p > 0.05$). Postmortem time did not affect the duration of retrieval (ANOVA: $F(3, 372) = 1.17, p = 0.32$). There was an interaction between the postmortem time and caste (ANOVA: $F(9, 372) = 2.38, p = 0.01$) (Figure 3.2C).

If a worker or nymph corpse was introduced 64h postmortem, nestmates tended to bury the corpse on site using sand, feces, paper fiber and/or any other materials they could find when they encountered the corpse. However, when a soldier corpse was introduced, in addition to burial behavior, termite workers exhibited wall-off behavior (using sand to block the entrance to the testing arena) or moved the corpse to the living chamber and then buried it (Figure 3.3A and 3.3B).

3.4.2 Chemical signature of corpses from different castes

3-Octanone and 3-octanol were detected in both worker and soldier corpses. These two volatile compounds, however, were absent or below detection limit in both types of nymph corpses. 3-Octanone reached the peak after 0.5h postmortem for both worker and soldier corpses, while 3-octanol reached the peak right after death in soldier corpses but

after 1h postmortem in worker corpses. These two volatiles gradually decreased after the peak, and became undetectable after 32 h. There were significant effects on the quantities in both volatiles, in castes (ANOVA; 3-octanone: $F(3, 959) = 385.05, p < 0.0001$; 3-octanol: $F(3, 959) = 199.74, p < 0.0001$), postmortem time (ANOVA; 3-octanone: $F(9, 959) = 136.30, p < 0.0001$; 3-octanol: $F(9, 959) = 65.37, p < 0.0001$) and their interaction (ANOVA; 3-octanone: $F(27, 959) = 53.69, p < 0.0001$; 3-octanol: $F(27, 959) = 28.70, p < 0.0001$). Worker corpses usually released significantly more 3-octanone than those of soldiers before 16 h (ANOVA; 0h: $F(24, 47) = 6.53, p = 0.014$; 0.5h: $F(24, 47) = 37.47, p < 0.0001$; 1h: $F(24, 47) = 22.00, p < 0.0001$; 4h: $F(24, 47) = 8.26, p < 0.01$; 8h: $F(24, 47) = 4.50, p < 0.05$); and released more 3-octanol than soldiers between 0.5 and 16h postmortem time (ANOVA; 0.5h: $F(24, 47) = 7.15, p = 0.01$; 1h: $F(24, 47) = 39.25, p < 0.0001$; 2h: $F(24, 47) = 12.73, p < 0.001$; 4h: $F(24, 47) = 9.72, p < 0.01$; 8h: $F(24, 47) = 6.54, p < 0.05$; 16h: $F(24, 47) = 6.05, p < 0.05$) (Figure 3.4A and 3.4B).

Indole and phenol were released by all castes 16h postmortem. There were significant effects on the quantities in both indole and phenol, among castes (ANOVA; indole: $F(3, 959) = 122.16, p < 0.0001$; phenol: $F(3, 959) = 62.33, p < 0.0001$), postmortem time (ANOVA; indole: $F(9, 959) = 7735.48, p < 0.0001$; phenol: $F(9, 959) = 2975.67, p < 0.0001$) and their interaction (ANOVA; indole: $F(27, 959) = 114.20, p < 0.0001$; phenol: $F(27, 959) = 58.03, p < 0.0001$). The accumulation of indole and phenol were different across castes after 16h (ANOVA; indole: $F(24, 95) = 5.18, p < 0.01$; phenol: $F(24, 95) = 5.12, p < 0.01$), 32h (ANOVA; indole: $F(24, 95) = 11.30, p < 0.0001$; phenol: $F(24, 95) = 3.54, p < 0.05$), 64h (ANOVA; indole: $F(24, 95) = 117.69, p < 0.0001$; phenol: $F(24, 95) = 64.03, p < 0.0001$) (Figure 3.4C and 3.4D). Oleic acid

accumulated as the corpse decayed in all four castes. The accumulation of oleic acid in soldier corpses was lower than in the other three castes across multiple postmortem times (ANOVA; caste: $F(3, 143) = 20.77, p < 0.0001$; postmortem time: $F(3, 143) = 297.50, p < 0.0001$; their interaction: $F(3, 143) = 13.15, p < 0.0001$). Significant differences of the oleic acid accumulation across castes showed in 16h (ANOVA; $F(9, 35) = 5.53, p < 0.01$), 32h (ANOVA; $F(9, 35) = 8.41, p < 0.001$), and 64h (ANOVA; $F(9, 35) = 15.40, p < 0.0001$), but not in live termites (ANOVA; $F(9, 35) = 0.069, p > 0.05$) (Figure 3.5). There was no colony effect on both early and late death cues (ANOVA; 3-octanone: $F(2, 959) = 0.14, p = 0.87$; 3-octanol: $F(2, 959) = 0.15, p = 0.86$; indole: $F(2, 959) = 0.02, p = 0.98$; phenol: $F(2, 959) = 0.07, p = 0.93$; oleic acid: $F(2, 143) = 0.03, p = 0.97$).

3.5 Discussion

3.5.1 Corpses from different castes elicit differential undertaking responses

The ability to recognize dead nestmates and respond to them properly is extremely important for social animals, particularly in the subterranean termites due to their high population density in an enclosed nesting environment. It is extremely challenging to quantify the natural turnover rate in eusocial insects, especially in the cryptic subterranean termites. Darlington (1991) documented the turnover in the field populations of a mound-building, fungus-growing termite, *Macrotermes michaelseni*. A daily production rate of 1.4% was estimated based on a pooled dataset from 22 mature nests over a span of seven years in Kenya, in which a total of 18,076 adults was produced on a daily basis from a mature *M. michaelseni* population containing 2,771,671 juvenile and 1,280,895 adult sterile termites (approximately 4.1 million individuals per colony without the foraging populations outside the nest, n=22, Darlington 1991). Assuming that

the population size of a mature colony is stable, we anticipate a similar daily death rate for *M. michaelseni*. In the case of *R. flavipes*, the most widely distributed termite species in North America (Austin et al. 2005), as many as five million individuals might be included in a single colony, covering over 2,000 square meters of foraging territory (Su et al. 1993). Although the colony size and caste composition are substantially different between the two species, a *R. flavipes* colony could, in theory, contain up to 70,000 nest mate corpses per day based on the 1.4% daily death rate in *M. michaelseni*. Encountering the dead is common and often in social insects, which makes corpse management a necessity (Sun and Zhou 2013). The purpose of corpse management is not only to provide a level of social immunity by limiting/eliminating the potential pathogenic risks associated with corpses (Cremer et al. 2007; Thorne 1991), but also to recycle nutrients (e.g., nitrogen) and possibly symbionts by cannibalizing dead conspecifics (Sun et al. 2017). In contrast to the eusocial Hymenoptera, for which removal is the primary means of corpse management (Wilson et al. 1958, Visscher 1983, McAfee et al. 2018), termite undertaking responses shift from cannibalism to burial depending on the dynamic change of early and late death cues (Sun et al. 2017).

In this study, corpses of all castes were carried inside the nest and cannibalized when the postmortem time was less than 64 hours. Cannibalism of freshly dead conspecifics has been reported in various termite species as a primary strategy to manage the corpse in order to recycle the nutrients (Sun et al. 2017, Neoh et al. 2012, Rosengaus and Traniello 2001, Chouvenc and Su 2012, Sun et al. 2018, Chouvenc 2020, Sun et al. 2020). Given that wood is notorious for its imbalance of carbon: nitrogen ratio (ranging from 75:1 to 247:1), consumption of freshly dead individuals would provide an important

nitrogen resource for wood-feeding termites (Hungate 1941, Sun et al 2017).

Theoretically, cannibalism on freshly dead conspecifics would also benefit termites by recycling gut symbionts that help them digest lignocellulose (Thorne 1990). Cannibalism of dead individuals in early stages of decomposition also protects the colony from pathogens associated with the corpse with the help of the antimicrobial properties of termite saliva and guts contents (Chouvenc et al. 2009, Rosengaus and Traniello 2001). Besides managing the corpses, termite has also been observed to cannibalize terminally ill nestmates as a collective hygienic behavior (Davis et al. 2018), attack and consume the wounded reproductives as a policing behavior (Sun et al. 2020), and recycle other live individuals as emergency food sources during a severe colony condition, such as starvation (Su and Lafage 1986).

When left to decompose further, the corpse would become too depleted of nutrients or risky to be recycled. Burial and associated behaviors then occur to block the decayed corpse from the rest of the healthy nestmates so as to protect the colony from the risks associated with corpses. In our results, burial behavior was consistently observed in highly decayed corpses regardless of castes. Additional behaviors, such as walling-off and movement of the corpse before burial were observed for 50% of soldier corpses. Under natural conditions, such circumstances could result from battle and competition, and dead soldiers would be generated from such agonism serving as an indicator of the potential threat. Walling-off of intrusion points results in these individuals being killed and left for decaying outside the colony, and may be rediscovered later. In *Coptotermes* termites, when two inter- or intraspecific colonies aggressively encounter one another, tunnel blockages are constructed (Li et al 2010, Lima and Costa-Leonardo 2012, Bernard

et al 2017). Similarly, an intricate undertaking response was documented in the Asian subterranean termite, *Coptotermes gestroi*, in which soldiers that had been dead for over 24 hours were buried, while freshly killed soldiers were buried, cannibalized (partially or fully), or ignored, demonstrating behavioral plasticity toward soldier corpses (da Silva et al. 2019). In addition, tunneling *C. gestroi* workers avoided areas containing dead soldiers from both conspecific and competitors (Lima and Costa-Leonardo 2012). Such “walling-off” behavior could potentially reduce the risk of subsequent aggression.

3.5.2 Chemical signatures are quantitatively and/or qualitatively different among corpses from different castes

Postmortem chemical profiles showed that the early death cues, 3-octanone and 3-octanol, in worker corpses were significantly higher than in soldier corpses, while they were undetectable in SWBN and LWBN corpses. In comparison, the late death cues, specifically oleic acid, were ubiquitously detected, however, these chemicals accumulated significantly slower in soldier corpses than other castes. The differential release of the early death cues between workers/soldiers and nymphs might be due to either quantitative or qualitative differences. Quantitatively, our detection method might not be sensitive enough to identify the trace-amount of 3-octanol and 3-octanone in nymph corpses. Qualitatively, there might be intrinsic differences in the biosynthesis of these two volatiles between imaginal and neuter developmental pathways. In addition, nymphs might use cues (e.g., tactile) other than chemicals to signaling death (Ulyshen and Shelton 2012). Such differences are the result of different chemical composition, physiology, biochemistry, etc., among castes.

Such differences in the early death cues lead to subtle changes in undertaking responses. Although freshly killed corpses were always cannibalized, it took longer for *R. flavipes* to locate and recycle nymph corpses lacking 3-octanone and 3-octanol. The early death cues help workers from the living chamber locate the corpses (Sun et al. 2017). However, the fact that nymph corpses were eventually cannibalized suggests the involvement of other sensory cues. Tactile cues have been suggested to synergize with oleic acid to elicit burial behavior in *R. virginicus* (Ulyshen and Shelton 2012). In comparison to castes in the imaginal line (nymphs), castes in the neuter line (workers and soldiers) carry out tasks with higher risks, including disease and predation (Cremer et al. 2007). Analogous to "immune privilege" documented in the germline or nerve cells in the central nervous system (Cremer et al. 2007, Janeway et al. 1999), reproductives in eusocial insects are expected to be subject to special protection. Nymphs, a reproductive-in-making caste, should share the social "immune privilege" with other castes within the imaginal line. In contrast, considered 'somatic' and disposable according to the "disposable soma" theory (Kirkwood 1987), the worker caste, especially for foragers (older workers), is more expendable. Consequently, the turnover rate should be lower in castes in the imaginal line (nymphs) compared to those in the neuter line (workers and soldiers). As such, a well-synchronized signaling system involving both early and late death cues allows *R. flavipes* to locate and respond to the corpses from the most risk-taking castes, i.e., workers and soldiers, quickly and efficiently (Sun et al. 2017). Meanwhile, the difference of accumulation in late death cues between the corpses of soldier and other castes could be proximately explained by 1) a delayed decomposition process, in which the accumulation of fatty acids was significantly slower in soldier

corpses than in other castes; and 2) the mix of defensive secretions in the postmortem chemicals in soldier corpses. Autolytic catabolism and bacterial hydrolysis of triglycerides contribute to the accumulation of fatty acids in dead bodies (Blum et al. 1970). With substantial modifications in body plan, morphology and chemistry in soldiers, such as enlarged head capsules and mandibles and defensive chemicals stored in the frontal gland (Noirot and Pasteels 1987), the decomposition processes and postmortem chemical profiles of soldiers are, understandably, different from those in workers. In addition, defensive secretions by soldiers protect the colony from intruders and pathogenic attacks (Prestwich 1979, Prestwich 1984, Zalkow et al. 1981). In a congeneric species, *R. speratus*, a soldier-specific volatile, (-)- β -elemene, exhibited fungistatic activity against entomopathogenic fungi (Mitaka et al. 2017). Fuller (2007) demonstrated that soldier secretions in *Nasutitermes acajutlae* inhibited the growth of fungi on freshly killed soldier corpses, suggesting a slower decomposition process in soldier corpses.

The possible causes of death in termites vary under natural conditions. In this study, we used a freeze-thaw method to generate termite corpses and examine their volatiles. Although freezing to death does exist in nature (personal observation), this method has been suggested to improve the detection of volatile compounds in several insect species, including *R. flavipes* (Chen 2017). Although we never observe any visible leaking of body fluid, i.e., hemolymph, from the corpses during the undertaking bioassay, ice crystals that are formed during the freeze-thaw cycle can cause cell membranes to rupture due to an imbalance in osmotic pressure. In a parallel study, however, death-related volatile compounds, including 3-octanol and 3-octanone, were ubiquitously

detected in *R. flavipes* workers dead from biotic (competition and disease) and abiotic (freezing, heat, desiccation, and insecticides) factors (unpublished data). That being said, how freezing affects the undertaking process, is a question warrants further investigation.

3.6 Summary and perspectives

Caste, division of labor, and well-orchestrated communication are key characteristics defining superorganisms. The assembly of superorganisms follows a series of algorithms, including developmental, behavioral, and genetic rules (Hölldobler and Wilson 2009).

The differential undertaking responses to corpses from different castes follow behavioral algorithms, in which the selection of behavioral responses differs between castes. In termites, cannibalism of freshly killed corpses benefits the colony by recycling nutrients, and potential gut symbionts and by preventing pathogens development that would occur if corpses were left unattended. Furthermore, burial and walling-off behavior can be used to separate highly decomposed corpses from the colony to prevent further disease transmission and avoid further contact with predators or competitors. Such differential responses are achieved in an autonomous, decentralized way (Pratt et al. 2002; Werfel et al. 2014) following the behavioral algorithms/rules (Hölldobler and Wilson, 2009).

Castes are created following the developmental algorithm, a series of sequential decision-making events guiding the growth of colony members to reach their respective developmental endpoints (Hölldobler and Wilson 2009). In *R. flavipes*, at the first decision point of post-embryonic development, the second instar larvae follow either the imaginal line to advance into nymphs (reproductives), or the neuter line to progress into workers and soldiers (non-reproductives). Death in the imaginal line is costly to the colony due to their all-important reproductive outputs, which warrants social “immune

privilege". Given that nymphs are typically nest-bound, risks associated with their allocated tasks and their turnover rate should be low. Indeed, the early death cues were absent or undetectable in nymphs. For the neuter line, however, the 'somatic' worker caste is disposable and replaceable. Workers carry out almost all tasks within the colony except reproduction. The downside with riskier tasks, such as foraging and undertaking, is the higher turnover rate in termite workers. Consequently, equipped with a battery of early and late death cues, termites are able to manage corpses from the numerically dominant worker caste in a timely and efficient manner to maximize colony fitness.

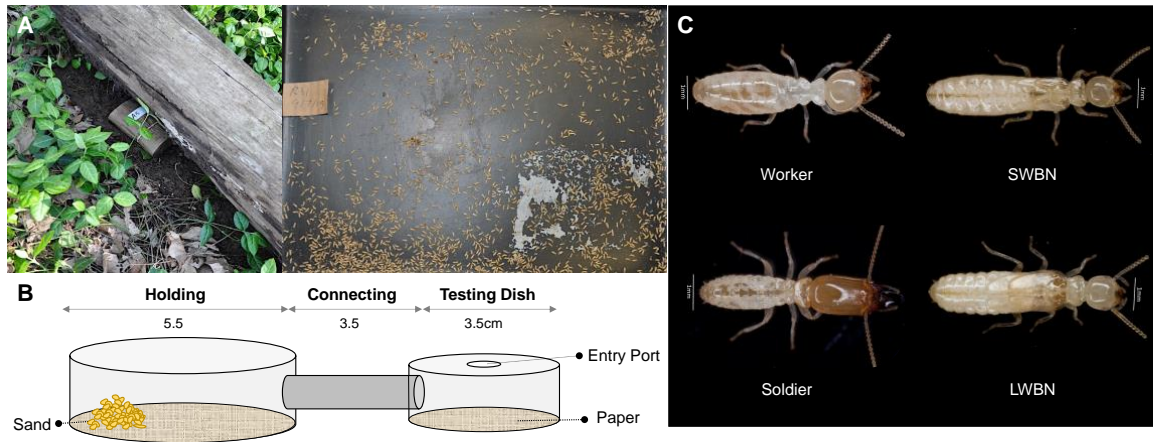


Figure 3.1 Termite collection, caste identification and experimental set-up.

Termite field collection. The left image represents a collection site, in which a termite trap was placed, and the right image shows termites were extracted from the cardboard traps and temporarily placed into a black PVC bucket for caste identification and measurement. Photo credit: Jizhe Shi. B) A schematic drawing shows experimental setup of the undertaking behavioral assay. C) Termite castes included in this study. Scale bar: 1mm. Photo credit: Li Tian.

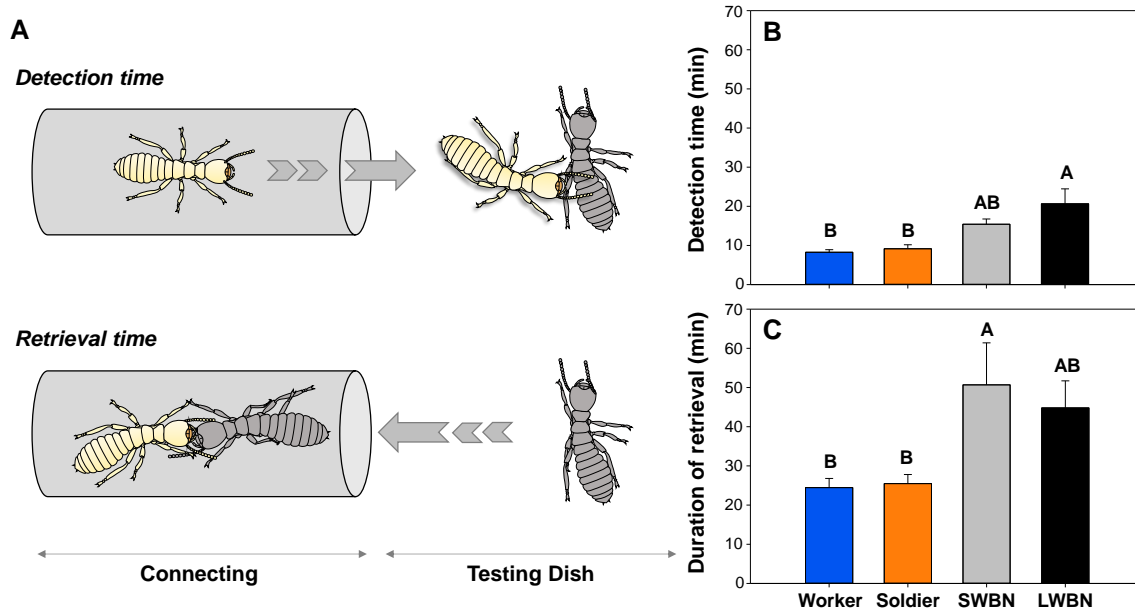


Figure 3.2 Behavioral responses to corpses with less than 64h postmortem time from different castes.

A) Schematic drawings show the definitions for the detection and retrieval time. The top drawing shows the first worker detected the corpse, and the bottom one depicts the corpse was considered retrieved when it was fully dragged into the connecting tube.

B) The first detection time of the introduced corpse (Mean + SEM, $n = 24$); C) The duration of retrieval of the corpse (Mean + SEM, $n = 24$). Data were pooled from 3 colonies with 8 replications for each colony. Means between groups denoted with the same letter were not significantly different (Tukey's HSD all-pairwise comparisons test, $P > 0.05$). Corpses were introduced at a series of postmortem times, including 0, 1, 16, and 32h.

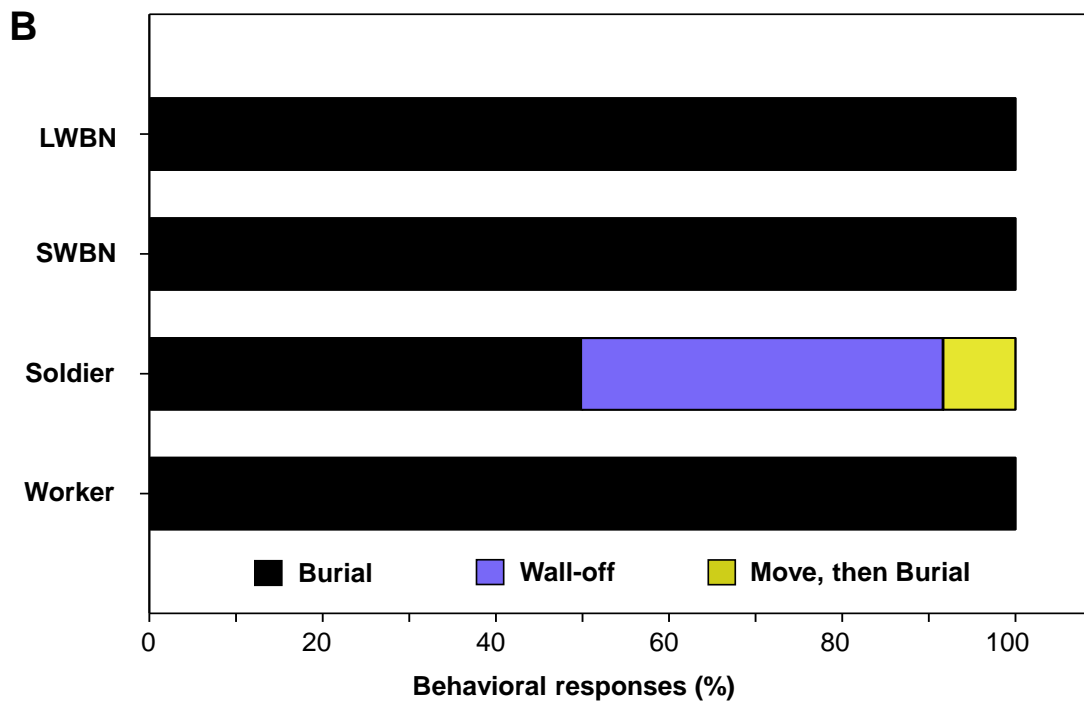
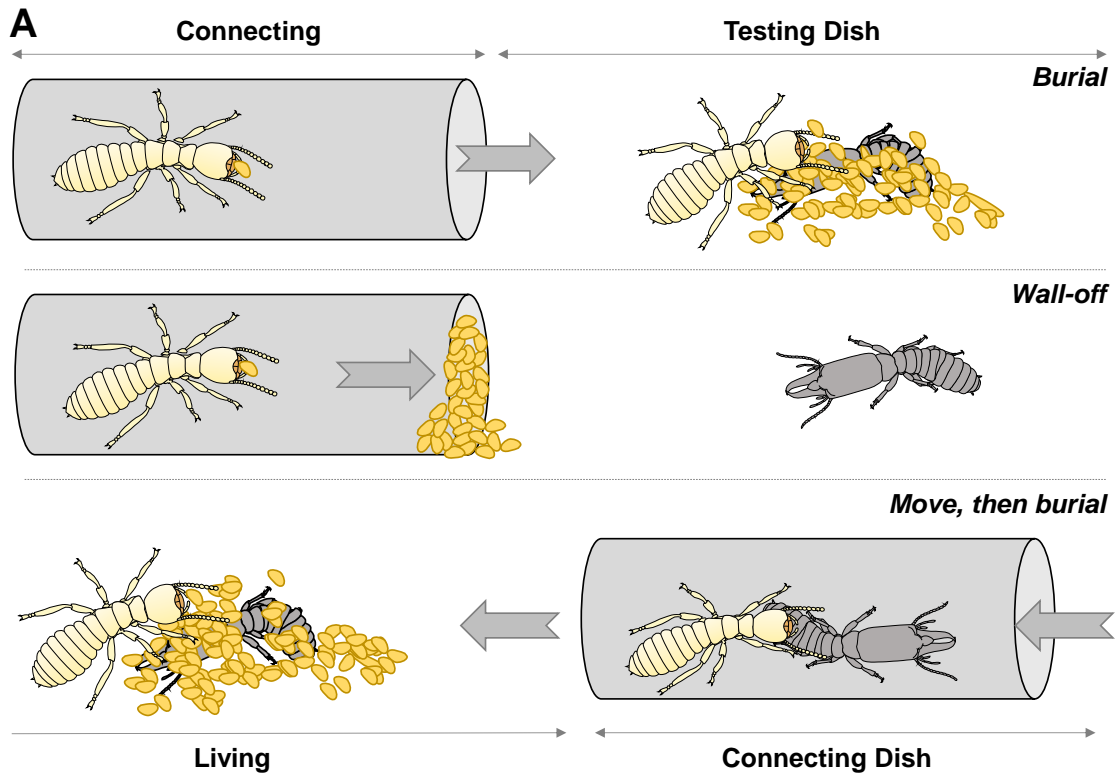


Figure 3.3 Different undertaking behavioral responses to 64 h postmortem corpses.

A) Schematic drawings depict three different undertaking behaviors when termites encounter corpses from different castes. Drawings from the top to the bottom represent

burial, wall-off, and move then burial, respectively. B) Behavioral responses to corpses from each caste. Colors represent different behavioral responses depicted in A. Data were pooled from 3 colonies with 8 replications for each colony.

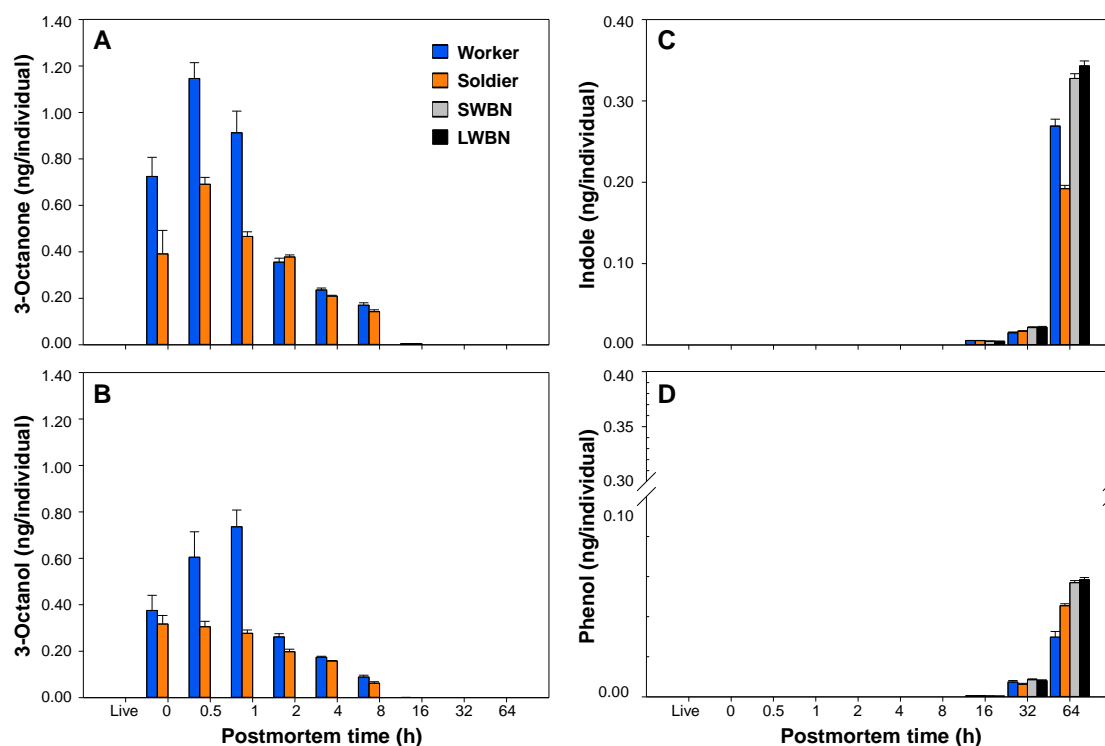


Figure 3.4 Temporal profiles of the early and late death cues associated with *R. flavipes* corpses from different castes.

A-D show the dynamic changes of the early (A: 3-octanone; B: 3-octanol) and late (C: indole; D: phenol) death cues. Quantities were determined by hexane extraction (Mean + SEM, n=24). Data were pooled from 3 colonies with 8 replications for each colony. Asterisks above the grouped bars denote significant difference (ANOVA): * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, NS: not significant.

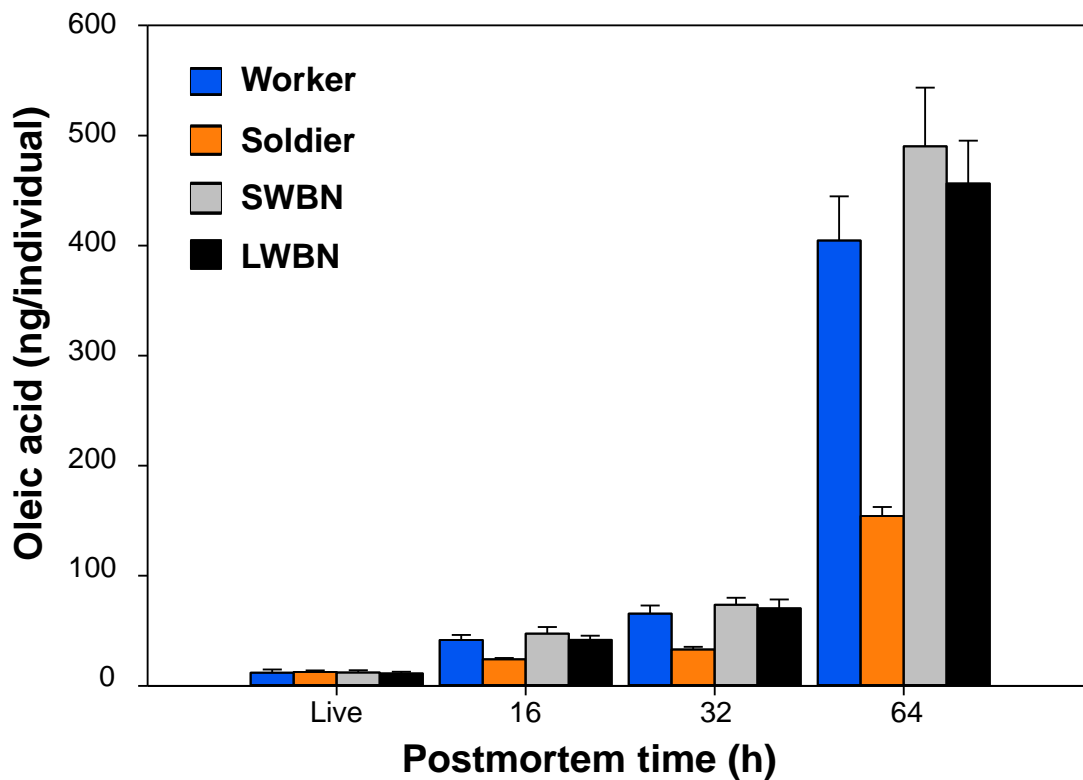


Figure 3.5 Temporal dynamics of oleic acid on *R. flavipes* corpses from different castes.

Different colors denote different castes. Quantities were determined by hexane extraction (Mean + SEM, n=9). Data were pooled from 3 colonies with 3 replications for each colony. Asterisks above the grouped bars denote significant difference (ANOVA): * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, NS: not significant.

CHAPTER 4. RELEASE MECHANISM OF EARLY DEATH CUES IN THE EASTERN SUBTERRANEAN TERMITE, *RETICULITERMES FLAVIPES*

4.1 Abstract

Chemical communication plays an important role in regulating undertaking behaviors in termites. The eastern subterranean termite, *Reticulitermes flavipes*, exploits two C8 volatiles, 3-octanol and 3-octanone, to locate and manage dead nestmates. The release mechanism, specifically the location (where) and timing (when), of these early death cues, is not fully understood in *R. flavipes*. Based on the biosynthesis pathways in plants and fungi, and our preliminary research, we hypothesized that 3-octanol and 3-octanone are synthesized in a general source, such as cuticle and/or hemolymph, prior to death.

To examine this hypothesis, we carried out the following objectives: 1) quantitatively investigated the spatial distribution of these compounds throughout different body parts and body fluids in *R. flavipes*; and 2) comparatively examined the release of 3-octanol and 3-octanone at ultra-low and room temperature conditions. Our results 1) confirmed the existence of 3-octanol and 3-octanone in the head, thorax, abdomen, and hemolymph; 2) detected higher concentrations of early death cues in the head and thorax than in the abdomen, suggesting the possible location of synthesis; and 3) revealed that ultra-low temperature did not affect the amount or concentration of early death cues across body parts, suggesting the synthesis of early death cues occurs prior to death.

This study offers the first peek into the release mechanism of early death cues in *R. flavipes* and suggests future research into functional characterization of these two compounds while termites are alive. Meanwhile, we also discussed the role of 3-octanol

and 3-octanone as a novel death cue during the evolution of termites and provided insight into comparative studies into the evolution of death-related chemicals for future studies.

4.2 Introduction

Chemical communication plays an important role in the evolution of eusociality, especially in regulating stereotypic behaviors and fundamental developmental processes, including foraging, reproduction, defensive behaviors, and undertaking behaviors (Blum, 1974; Wilson, 1971; Hölldobler and Wilson, 1990; Sun et al., 2013). Eusocial insects have evolved to exploit death-related chemicals to detect their dead conspecifics and respond accordingly (Wilson et al., 1958; Sun et al., 2013; Gonçalves and Biro, 2018). Death-related chemicals can be categorized based on the time of their production, i.e., chemicals synthesized before or after death (Sun et al., 2018). For chemicals produced after death, the accumulation of fatty acids as a death cue was first demonstrated by E. O. Wilson and his colleagues in 1958 in two ants, *Pogonomyrmex badius* and *Solenopsis saevissima*, in which oleic acid was used as a death cue to induce corpse removal (Wilson et al., 1958). For chemicals produced prior to death, chemicals are actively synthesized and accumulated when the insects are still alive, with changes in either function or quantity occurring after death. For example, in the Argentine ant, *Linepithema humile*, the vanishing of two chemicals associated with life, dolichodial and iridomyrmecin, happens right after death and reveals the corpse removal cue (Choe et al., 2009). In the honeybee, *Apis mellifera*, β -ocimene, which is used as a food-begging signal in living larvae, continues to be released after death and recruits workers to uncap and remove dead larvae (McAfee et al., 2018).

Termites have developed sophisticated corpse management strategies primarily based on chemical cues. Accumulated fatty acids with a combination of two decomposition-related volatiles, phenol and indole, have been broadly found to induce burial behavior in termites (Chouvenc et al., 2012; Ulyshen and Shelton, 2012; Sun et al., 2017). In the eastern subterranean termite, *Reticulitermes flavipes*, two novel C8 volatiles, specifically 3-octanol and 3-octanone, were named as early death cues and found to be released immediately after death to recruit nestmate workers for cannibalism (Sun et al., 2017). The interplay between rapid postmortem release and diminishment of early death cues and gradual accumulation of late death cues (phenol, indole, and fatty acids) is suggested to be responsible for the behavioral switch from cannibalism to burial to balance the nutritional benefits and risks associated with conspecific corpses (Sun et al., 2017). However, the case of early death cues in *R. flavipes* is isolated. There is still much unclear about these compounds in *R. flavipes*, such as the release mechanism, in particular, the location and timing of 3-octanol and 3-octanone synthesis.

3-Octanol and 3-octanone are widely recognized in plants (Kihara et al., 2014) and fungi (Combet et al., 2006) as being responsible for flavor, chemical defense, and fungal growth inhibition (Combet et al., 2006; Hung et al., 2015; Pennerman et al., 2021). In plants and fungi, fatty acids, specifically linoleic acid, are suggested to be precursors in the biosynthesis of the C8 volatiles in an enzyme-catalyzed process (Kihara et al., 2014; Combet et al., 2006). The synthesis pathway of 3-octanol and 3-octanone in *R. flavipes* is most likely an enzymatic process. For the location of synthesis, these chemicals may come from either an exocrine gland, as are the monoterpenes secreted by the frontal glands of termite soldiers that function as alarm pheromones (Pasteels and Bordereau,

2019; Billen and Morgan, 2019), or a general source, as are the cuticular hydrocarbons used in nestmate recognition (Dani et al., 2005; Billen and Morgan, 2019; Costa-Leonardo and Hafig, 2010). Our preliminary research detected these two compounds in the hemolymph of *R. flavipes* workers, implying that synthesis occurs in a general source. For the timing of synthesis, 3-octanol and 3-octanone are emitted right after death in *R. flavipes*, which is similar to the quick emission of C8 volatiles in plants and mushrooms following tissue damage. Rapid synthesis based on pre-existing precursors and enzymes when death occurs might account for this timely release. As an alternative, these substances may be synthesized while the termites are still alive and released after death. In our preliminary study, 3-octanol and 3-octanone were detected in injured *R. flavipes* workers, which suggested that synthesis might happen before death.

Based on the biosynthesis pathways of 3-octanol and 3-octanone in plants and fungi and preliminary research, we hypothesized that these compounds are synthesized in a general source, such as cuticle and/or hemolymph, before death. To test this hypothesis, we have conducted the following objectives: 1) quantitatively examined the spatial distribution of 3-octanol and 3-octanone across different body parts and body fluids in *R. flavipes*; and 2) comparatively quantified the release of the compounds during ultra-low and room temperature treatments. If the compounds originate from a general source, such as cuticle and/or hemolymph, distribution of 3-octanol and 3-octanone should be consistent with the distribution of these sources. As a suggested enzymatic process, if biosynthesis of 3-octanol and 3-octanone happens before death, the ultra-low temperature-induced suppression of synthesis-related enzyme activity should not affect the release of these chemicals.

4.3 Material and methods

4.3.1 Termite collection and maintenance

Three termite colonies were all collected from Kentucky, USA. Two colonies of *R. flavipes*, RI-31 and RII-3, were collected from two distinct collecting sites in Wildcat Trail in the Red River Gorge area inside the Daniel Boone National Forest (Kentucky, US). One other colony (KY47) was collected from a park in Christian County, Kentucky. All three colonies were collected during the spring collecting season in 2022 and used within 6 months of collection. Colonies were captured by putting cardboard rolls under fallen wood logs. Cardboard rolls were replaced every one to two weeks. Termites were extracted from collected rolls and quarantined for 14 days in the lab, then transferred into a plastic box with other termites collected from the same site as a colony. Moistened pine wood pieces and mulch (Lowe's Companies, Inc. NC, US) were provided in the plastic box as food sources. Colonies were kept in 24-hour darkness at $27\pm 1^{\circ}\text{C}$ with 80-90% RH. Before use in the experiments, termites were lured from the plastic boxes using moistened cardboard rolls (diameter: 5 cm; height: 15.2 cm) and stored in Petri dishes (145W x 20H mm, VWR International, LLC., PA, US) provisioned with a layer of unbleached paper towel moistened with distilled water.

4.3.2 Analysis of 3-octanol and 3-octanone with different body parts and fluids in *R. flavipes*

Qualitative analysis using head-space SPME

To find the source of early death cues in *R. flavipes*, the presences of 3-octanol and 3-octanone were tested for in three dissected body parts (head, thorax, abdomen) from different castes (worker, soldier and nymph) as well as injured individuals using

headspace SPME (Solid phase microextraction). Three types of body fluids potentially secreted during death in workers (hemolymph, as well as secretions from the mouthparts and rectum) were also tested using SPME. Living termite individuals were used as controls. Samples were prepared under room temperature (26°C).

Body parts from different castes

Three castes from *R. flavipes*, worker, soldier and short-wing bud nymph (SWBN), were selected to test the existence of two early death cues in different body parts. Termites were placed in a 3.5 cm Petri dish, held with forceps (Bioquip Inc, Compton, CA, US) and dissected into head, thorax and abdomen with micro-dissecting scissors (Electron Microscopy Sciences, PA, US) under a dissecting microscope (SMZ745T, Nikon, Tokyo, Japan). Dissected body parts were moved into a 0.1 mL glass v-vial (DWK Life Sciences, Millville, NJ) right after the cut. The vial was sealed with an aluminum cap with PTFE/red rubber immediately after transferring the body part or individual. Scissors and forceps used for cutting and transferring body parts were cleaned with ethanol between samples. Injured individuals were generated by carefully making a non-fatal cut in the lower abdomen of living individuals, then stored following the same procedure as used with body parts. Three replications were prepared across three castes for each of the three colonies.

Body fluids from workers

Hemolymph samples were prepared from groups of 50 termite workers per sample using the following procedure. The dorsal side of each worker was stuck to a piece of adhesive

tape (VWR International, LLC., PA, US). One of the hind legs was carefully removed with micro-dissecting scissors under the dissecting microscope. Hemolymph droplets bled from the wound were quickly collected with a 10 μ L micro pipet (Fisher Scientific, Pittsburgh, PA) and transferred into a 0.1 mL glass v-vial (DWK Life Sciences, Millville, NJ) for later VOC analysis. Three replications were prepared for each colony.

Mouthpart and rectum secretions were obtained from 50 termite workers for each sample. Each termite worker was placed on the lid of a 3.5 cm Petri dish (VWR International, LLC., PA, US), then another lid was placed onto the termite's body and carefully pressed. Liquid from mouthpart and rectum was transferred into a 0.1 mL glass v-vial (DWK Life Sciences, Millville, NJ) with a 10 μ L micro pipet (Fisher Scientific, Pittsburgh, PA). Three replications were made with secretions from either mouth or rectum for each of the three colonies, respectively.

Head-space SPME

Volatiles released from body parts and injured individuals from different castes and body fluids from workers were extracted via head-space SPME. A 100 μ m SPME fiber with Polydimethylsiloxane phase (Agilent Technologies, Santa Clara, CA, USA) was carefully inserted into the glass vial and extended onto the headspace of the samples without contact. Samples were extracted for 10 min.

Quantitative analysis of worker body parts

To locate the potential source of early death cues, 3-octanol and 3-octanone were quantified in different body parts of *R. flavipes* workers. Workers were held with forceps

in a 3.5 cm Petri dish under a dissecting microscope, then dissected into head, thorax and abdomen under room temperature (26°C). Each body part was immediately transferred into a glass conical vial (0.1 mL) containing 50 µL hexane with 10 ng/µL alkane hydrocarbon, *n*-nonadecane (Sigma-Aldrich, St. Louis, MO, US) used as an internal standard for quantification. Vials were sealed with an aluminum cap lined with PTFE/red rubber (DWK Life Sciences, Millville, NJ, US). Extraction lasted for 10 min. The supernatant was transferred into another 0.1 mL conical vial with an aluminum cap. Two µL of extractions were injected into the GC machine for chemical analysis. Six replications were conducted for each of the three colonies.

GC-MS

Samples were injected into GC-MS (Gas Chromatography-Mass Spectrometry, Agilent Technologies, Santa Clara, CA, USA) for chemical analysis. The SPME fiber was injected into the GC through an inject port at 280°C for 1min. For samples extracted with hexane, 2 µL of extractions were injected into GC. The split-less mode gas chromatograph was equipped with a 30 m × 0.25 mm × 0.25 µm DB-5 capillary column and helium was used as a carrier gas. The start temperature of the column was set as 40°C. Temperature was programmed for 2 min holding and then to increase by 10°C per minute to 320°C. Compounds were identified with an MS (Mass Spectrometer, Agilent Technologies 5975, Santa Clara, CA, USA) using the National Institute of Standards and Technology/National Institutes of Health/Environmental Protection Agency Mass Spectral Library.

4.3.3 Comparing the release of 3-octanol and 3-octanone in different body parts of workers between ultra-low and room-temperature treatments

To determine the timing of biosynthesis of 3-octanol and 3-octanone in *R. flavipes* workers, compounds were quantified in different body parts under ultra-low temperature simultaneously with those observed under room temperature in the previous section.

Workers were dissected and extracted with hexane using the same procedure described in the previous section, except under ultra-low temperature conditions. Specifically, workers were placed in a 3.5 cm Petri dish on a laboratory chill table (Temperature: -10°C, Bioquip Inc, Compton, CA, US) before dissection. Termites were held with forceps while 20 mL of liquid nitrogen was poured into the Petri dish. Termites were carefully cut into head, thorax and abdomen while liquid nitrogen remained. Glass conical vials (0.1 mL) were filled with 50 µL distilled hexane with an internal standard (10 ng/µL), *n*-nonadecane (Sigma-Aldrich, St. Louis, MO, US), before the dissection. Immediately following the dissection, each body part was put into a prepared glass conical vial. After a 10 min extraction, supernatant was transferred into a new 0.1 mL glass conical vial. Two µL of the supernatant was used in GC-MS analysis. The GC-MS was programmed identically as described in the previous section. Six replications were conducted for each of the three colonies. Data retrieved were used for comparison with data from room-temperature treatments.

4.3.4 Data analysis

Data analysis was performed using JMP® Pro 14.0.0 (SAS Institute Inc., NC, US). Before conducting ANOVA, normality was assessed. Amounts of 3-octanol and 3-octanone were

log-transformed to fulfill the assumptions of the parametric analysis. A two-way ANOVA was used with different body parts, temperature treatments, and their interaction as factors. Tukey's HSD all-pair-wise analysis was utilized following the ANOVA to compare the amounts of chemicals across different body parts under ultra-low and room temperature treatments. All figures were generated by PRISM Version 9.1.2 (225) (GraphPad Software, LLC., CA, US).

4.4 Results

4.4.1 Spatial distribution of 3-octanol and 3-octanone in *R. flavipes*

The existence of early death cues (3-octanol and 3-octanone) was tested in dissected body parts of different castes in *R. flavipes* (worker, soldier and SWBN) and body fluids (hemolymph, secretions from mouthparts and rectum) of workers (Figure 4.2). Early death cues were detected in both injured *R. flavipes* workers and soldiers and their dissected body parts. No early death cues were detected in living termites from any caste. These two volatiles were further detected in hemolymph of workers but not in secretions from either the mouthparts or rectum (Figure 4.3).

Dissected body part did not show a significant effect on the detected amount of either early death cue (3-octanol: $F_{2,106} = 0.13$, $p = 0.89$; 3-octanone: $F_{2,106} = 1.48$, $p = 0.23$). For releasing amount by unit, body part showed a significant effect on the detected amount of both compounds (3-octanol: $F_{2,106} = 3.61$, $p < 0.05$; 3-octanone: $F_{2,106} = 4.17$, $p < 0.05$). The detected amount of each compound was then compared across body parts. No significant differences were found in the detected amount of 3-octanol across body parts ($F_{2,48} = 0.16$, $p = 0.86$), nor of 3-octanone ($F_{2,50} = 0.36$, $p = 0.70$). When normalized by unit for each body part, 3-octanone was detected to be released significantly more by

unit in head and thorax than abdomen ($F_{2,50} = 6.33$, $p = 0.004$), whereas no significant difference was found in unit-wise 3-octanol across body parts ($F_{2,48} = 3.17$, $p > 0.05$).

4.4.2 Release of 3-octanol and 3-octanone under ultra-low and room temperature treatments

Early death cues were quantified in three dissected body parts (head, thorax and abdomen) in termite workers under ultra-low and room temperature treatments. Unit-wise, early death cues were found to be released more from heads and thoraxes than from abdomens (Figure 4.4). Ultra-low temperature treatment did not cause any significant change in the releasing amounts of 3-octanol and 3-octanone across different body parts except for 3-octanone from the heads (Figure 4.4).

Temperature showed no significant effects on the detected amount of either early death cue (3-octanol: $F_{1,106} = 0.002$, $p = 0.96$; 3-octanone: temperature: $F_{1,106} = 0.23$, $p = 0.64$) with no interactions with body parts (3-octanol: $F_{2,106} = 0.008$, $p = 0.99$; 3-octanone: $F_{2,106} = 0.84$, $p = 0.44$). For releasing amount by unit, temperature still showed no significant effect with no interaction with body part ($p > 0.05$).

Release of 3-octanol and 3-octanone was then compared between ultra-low and room temperature treatments in each body part. Only 3-octanone released by heads was detected to be higher under room temperature than under ultra-low temperature in terms of both detected amount and amount normalized by unit ($F_{1,34} = 7.29$, $p = 0.011$), while no other significant difference in 3-octanol and 3-octanone was detected in any body part between the two temperature treatments ($p > 0.05$).

As in the detected amounts of 3-octanol and 3-octanone across body parts under room temperature, no significant difference of these compounds was found under ultra-low temperature (3-octanol: $F_{2,50} = 0.82$, $p = 0.44$; 3-octanone: $F_{2,52} = 0.85$, $p = 0.43$). When normalized by unit for each body part, the distribution of both compounds showed a similar trend as observed under room temperature. Both 3-octanol and 3-octanone were detected to be released significantly more by unit in head and thorax than abdomen under ultra-low temperature (3-octanol: $F_{2,50} = 6.76$, $p = 0.003$; 3-octanone: $F_{2,52} = 5.67$, $p = 0.006$).

4.5 Discussion

4.5.1 Synthesis of 3-octanol and 3-octanone potentially occurs in head and thorax in *R. flavipes* workers

Subterranean termites live in enclosed nesting structures (Wilson et al. 1958, Shelton 2014, Sun and Zhou 2013). Timely release of volatile early death cues, 3-octanol and 3-octanone, in the eastern subterranean termite, *R. flavipes*, contribute to the location of recently deceased corpses by other workers (Sun et al., 2017). Our study showed that the early death cues are present in hemolymph, injured workers, and all dissected body parts in *R. flavipes*. These results imply that in *R. flavipes*, injured workers and any dismembered body parts are capable of raising alarm (Reinhard et al., 2003) and attracting other workers to the "accident scene" for later management when the colony is attacked (Sun et al., 2017).

Quantification of 3-octanol and 3-octanone across body parts suggested that the potential source of the early death cues is within the head and thorax. We initially found that the head, thorax, and abdomen released similar amounts of 3-octanol and 3-octanone.

Similarly, in Reinhard et al., 2003, the authors mentioned unpublished data showing that compound composition, including 3-octanol and 3-octanone, showed no difference between body parts. The head and thorax generally weigh less than the abdomen in termite workers. By normalizing releasing amount with body part weight, our results showed that the head and thorax in general possess more 3-octanol and 3-octanone by unit compared with the abdomen, implying that their distribution in the termite body is concentrated in the head and thorax.

Although our results imply that these chemicals are concentrated in the head and thorax and this distribution is in distant contrast with the distribution of hemolymph, the possibility of hemolymph as the source of early death cues cannot be excluded. By using C14 labeled polyethylene glycol as a marker, the volume of hemolymph was found to be highest in the abdomen and lowest in the head at 22-25°C in the honeybee, *Apis mellifica*; however, the distribution of hemolymph is largely influenced by treatment temperature, moving from abdomen to head and reaching a similar level as thorax when treated with lower temperatures (Crailsheim, 1985). Death is a complex process and is accompanied with severe conditions (Sun et al., 2018). How the death process may affect the distribution of hemolymph in termites remains unclear. Moreover, the distribution of hemolymph might not be homogeneous in *R. flavipes*. Although hemolymph circulates throughout the insect body along with hemocytes, not all hemocytes circulate with hemolymph. For example, in the malaria mosquito, *Anopheles gambiae*, sessile immune cells (hemocytes) concentrate on the outer surface of the dorsal vessel rather than circulating with hemolymph flow (King and Hillyer, 2013). Though it is clear from our study that the source of early death cues is located in the head and thorax as a general

tissue or exocrine gland, determining the exact source of synthesis and release of 3-octanol and 3-octanone requires further investigations.

4.5.2 Early death cues are suggested to be synthesized prior to death

Similar to the rapid emission of C8 volatiles in plants and mushrooms after tissue injury, 3-octanol and 3-octanone are released immediately after death in *R. flavipes*. This timely release could be explained by rapid biosynthesis of pre-existing precursors and enzymes when death occurs. Alternatively, these compounds could be produced while the termites are still alive and released when death happens. Under the ultra-low temperature treatment, slower-moving molecules cause fewer collisions between enzymes and substrates, which lowers enzyme activity. Our study demonstrated that ultra-low temperatures did not affect the amount of 3-octanol and 3-octanone in each body part, suggesting that the time of their biosynthesis occurs prior to death in *R. flavipes*. In plants and fungi, fatty acids may serve as precursors in the enzyme-catalyzed process of C8 volatile production (Kihara et al., 2014; Combet et al., 2006). The major C8 volatile in liverwort, *Marchantia polymorpha*, is 1-octen-3-ol, which is further modified into 3-octanone and 3-octanol by acetylation and oxidoreduction (Kihara et al., 2014). In the soybean, *Glycine max*, 3-octanyl β -primeveroside has been identified as a precursor of 3-octanol during soy soaking (Feng and Hua, 2022). Non-enzymatic synthesis of 3-octanol and 3-octanone usually requires a higher temperature (over 45°C) under artificial conditions (Xu et al., 2019; Kihara et al., 2014). Biosynthesis of 3-octanol and 3-octanone in *R. flavipes* is most likely an enzymatic process occurring before death.

Social insects primarily rely on chemicals released from dead conspecifics (death cues) to elicit sophisticated behavioral responses (Wilson et al., 1958; Sun et al., 2013; Gonçalves and Biro, 2018). Death cues are suggested to be divided into two groups according to the time of biosynthesis, i.e., chemicals produced while the animals are alive (prior to death) and chemicals produced during the decomposition of the corpse (after death) (Sun et al., 2018). Our study suggests that 3-octanol and 3-octanone, early death cues in *R. flavipes*, are synthesized prior to death. Another example of death cues synthesized prior to death is found in the Argentine ant, *Linepithema humile*, where dolichodial and iridomyrmecin, which are present on the cuticle, are synthesized in the pygidial gland while the ants are alive. The disappearance of these two substances shortly after death reveals the true corpse removal cue, triglycerides (Choe et al., 2009). An additional example is a brood pheromone called β -ocimene that has been found to act as a food-begging signal in honeybee larvae while they are alive and continues to be released after death, which attracts workers to remove the larval corpse (McAfee et al., 2018). Although it has long been established that chemicals created during decomposition play a significant role in corpse management in eusocial insects, it is insufficient to explain the quick reactions to recent conspecific death. (Wilson et al., 1958; Sun et al., 2013; Gonçalves and Biro, 2018). For example, in the red imported fire ant, *Solenopsis invicta*, and honeybee, *Apis mellifera*, corpses are removed within an hour of death (Howard et al., 1975; Visscher, 1983). Similarly, in *R. flavipes*, freshly killed corpses are retrieved back to the colony within 30 minutes (Sun et al., 2017). Although examples of death cues in the "before death" category are still uncommon and isolated, they help to explain the social insects' quick reactions to deceased conspecifics.

4.5.3 Insight into functions and evolution of early death cues

The next question would be what the functions of 3-octanol and 3-octanone are when a termite is still alive. Despite the fact that our findings suggest these two chemicals are produced prior to death, SPME extraction failed to detect these two substances in any living termites. This result implies that these two volatiles serve other purposes when the termites are alive besides serving as death cues, and that they are not used to communicate with colony members while the individual is still alive (Costa-Leonardo and Haifig, 2010; Billen and Morgan, 2019).

In *R. flavipes*, ergatoids, neotenic reproductives developed from workers, compete for reproductive rights. When one ergatoid attacks a counterpart, a cuticle-broken wound results, followed by hemolymph leaking. Other workers are then recruited to cannibalize the injured ergatoid (Sun et al., 2020). Our study traced 3-octanol and 3-octanone in hemolymph, however, further research is necessary to determine if 3-octanol and 3-octanone from hemolymph are activated during this policing behavior. Fatty acid oxidation in fungi and plants results in the production of eight carbon volatiles, which serve as antimicrobial agents against bacteria and fungi (Combet et al., 2006; Hung et al., 2015). In the banana pseudostem weevil *Odoiporus longicollis*, 3-octanone can be detected when weevils are infested by an entomopathogenic fungus, *Metarhizium robertsii* (Viswakethu, et al., 2022). This observation suggests a potential individual-level immune function of 3-octanol and 3-octanone in *R. flavipes*.

As in our previous research, 3-octanol and 3-octanone were found to be released by worker and soldier corpses in *R. flavipes* but not by short-wing bud nymphs. (Shi et

al., 2021). The lack of early death cues in the imaginal line (nymphs) is an adaptation to the specific ecological niche of termites. In terms of longevity, nymphs rarely die before developing into reproductives, i.e., nymphoids or alates. Nymphs stay in the center of the nest with a special "social immune privilege" in contrast to castes on the neutral line (workers and soldiers), who handle tasks with higher inherent risks of death. (Cremer et al. 2007). Regardless of the absence of 3-octanol and 3-octanone, recently killed nymph corpses are still retrieved and cannibalized (Shi et al., 2021). Workers may be able to locate and manage nymph corpses using cues other than chemical cues, such as tactile cues (Ulyshen and Shelton, 2012).

To date, documentation of 3-octanol and 3-octanone in termites is still rare and restricted to *Reticulitermes* termites (Reinhard et al., 2003; Sun et al., 2017). These compounds have also been identified from the whole-body extracts of workers in three congeneric termites, *R. lucifugus*, *R. grassei*, and *R. banyulensis*, alongside *R. flavipes* (the species was previously demonstrated as *R. santonensis* during the time of this study, but has since been understood as a synonym of *R. flavipes* (Austin et al., 2005)) (Reinhard et al., 2003). However, these compounds could not be found in the frontal gland secretions of soldiers in seven *Reticulitermes* sp., *R. santonensis* (now *R. flavipes*), *R. grassei*, *R. lucifugus*, *R. banyulensis*, *R. balkanensis*, *R. lucifugus corsicus* and a novel *Reticulitermes* species (Quintana et al., 2003). In these studies, secretions from the frontal gland pore of soldiers were carefully collected using a small piece of filter paper and extracted with n-pentane. Soldiers were not killed by solvent extraction as workers were, which might explain the absence of these two compounds. Preliminary chemical profiling of 3-octanol and 3-octanone in related animal taxa also detected these two compounds in

another congeneric termite, *R. mallei*, but failed to identify them in a dampwood termite, *Zootermopsis nevadensis*, and a woodroach, *Cryptocercus darwini* (Shi, unpublished data). *Zootermopsis nevadensis* belongs to the family Archotermopsidae and is considered a more ancestral termite species than *R. flavipes* (Rhinotermitidae), whereas the *Cryptocercus* roaches are the closest relatives to termites derived from the same wood-feeding roach ancestor (Bucek et al., 2019; Chouvenc et al., 2021). Moreover, these two compounds have also been documented in ants as an alarm pheromone emitted from the mandibular gland, including 3-octanone in *Manica mutica* and *M. bradleyi* (Fales et al., 1972), and both 3-octanol and 3-octanone in *Crematogaster* (Crewe et al., 1972) and *Camponotus* ants (Duffield and Blum, 1975). In addition, in *Ultracoelostoma* spp., volatiles, including 3-octanol, have been identified in honeydew and have been suggested to attract native birds and other insects (Brown et al., 2015). The emergence of 3-octanol and 3-octanone biosynthesis and their employment as early death cues is most likely a novel trait within *Reticulitermes* to rapidly locate dead conspecifics and retrieve their nutrition. This particular trait might contribute to the success of *Reticulitermes*, which must otherwise cope with a diet low in nitrogen. Comparative investigations of early death cues in other termite taxa should be encouraged to pursue a better understanding of the evolutionary history of death-related chemicals. Our study of the synthesis of early death cues may also suggest future studies into the functional characterization of these two volatiles in other pathways in live termites.

4.6 Summary

Although found in a variety of taxa, 3-octanol and 3-octanone have only been confirmed to operate as early death cues in corpse management in the eastern subterranean termite,

R. flavipes. To achieve a better understanding of their release mechanism, we used GC-MS for chemical analysis to test the hypothesis in *R. flavipes* that 3-octanol and 3-octanone are synthesized in a general source before death. Taken together, our results revealed that 1) 3-octanol and 3-octanone are distributed throughout the entire body cavity in *R. flavipes*, including the head, thorax, abdomen, and hemolymph; 2) higher concentrations of these compounds were detected in the head and thorax than in the abdomen, implying the possible location of synthesis; and 3) their amounts or concentrations across body parts were not affected by ultra-low temperature, suggesting that synthesis occurs before death. The significance of 3-octanol and 3-octanone as a novel death cue during the evolution of termites was also discussed, and we offered some insight into comparative investigations into the evolution of death-related chemicals for future research. Our study represents the first step in understanding the release mechanism of these compounds in *R. flavipes*, however, much remains to be investigated in future studies, for instance, functional characterization of these two compounds while termites are alive.

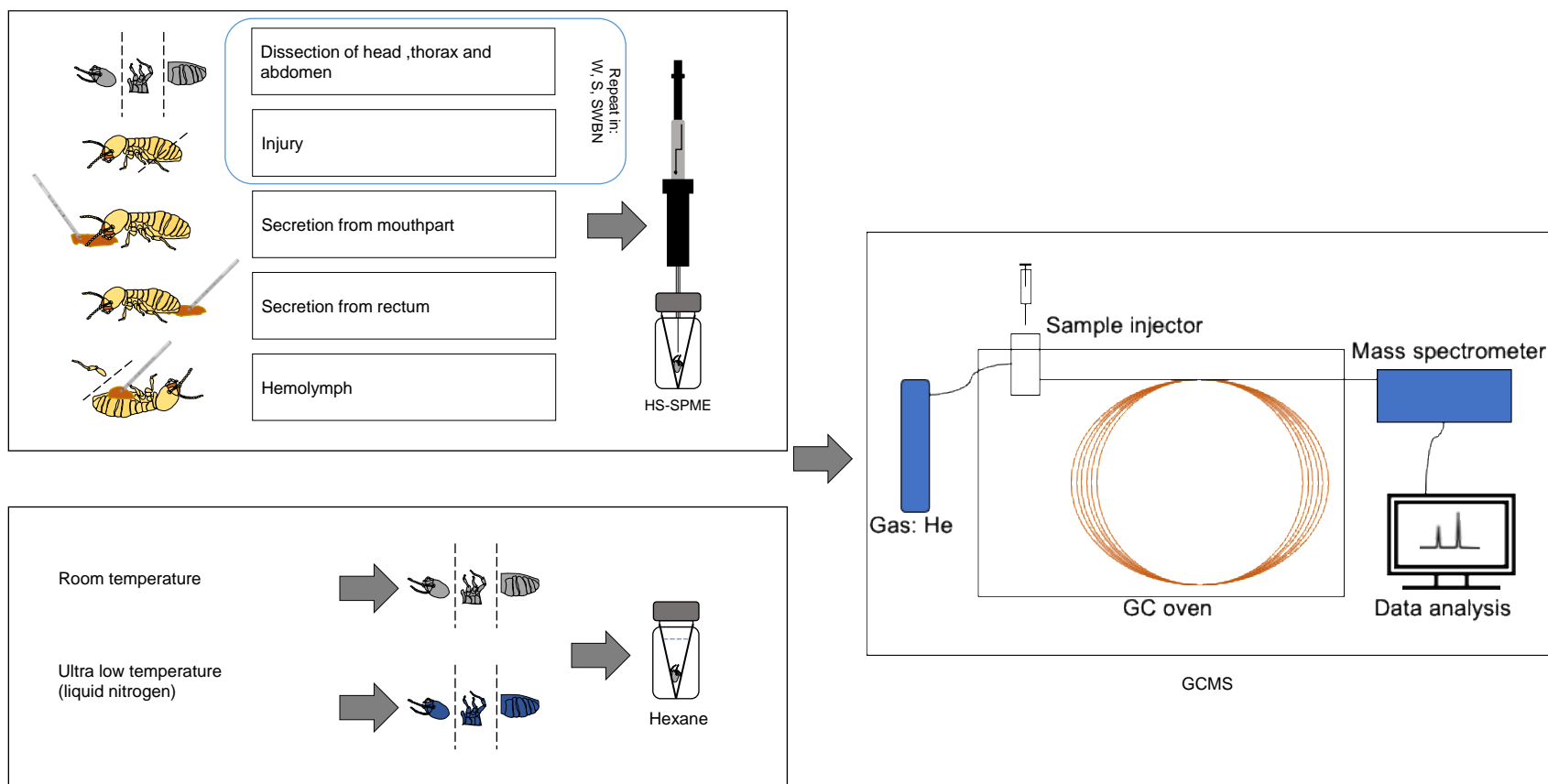


Figure 4.1 Workflow of sample preparation and experimental set-up.

Process of sample collection (HS-SPME and hexane extractions, respectively) and experimental setup was demonstrated by schematic drawing.

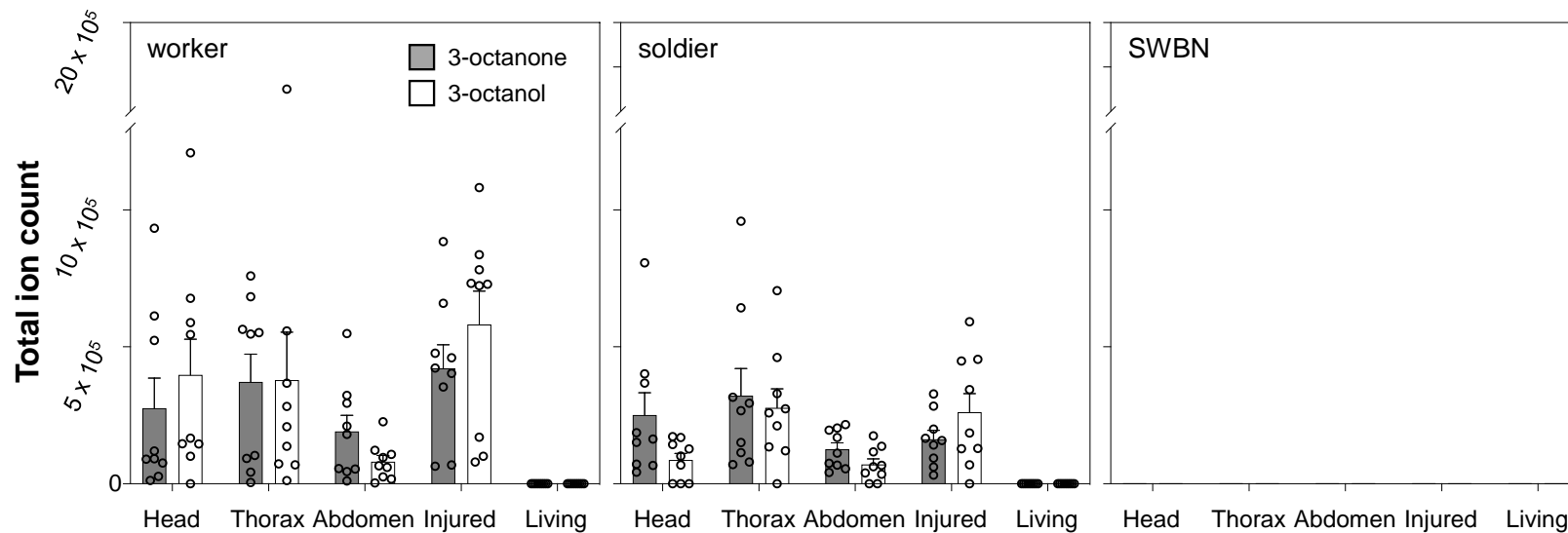


Figure 4.2 Screening potential sources of early death cues from different body parts in different termite castes.

Panel of bar plots represents HS-SPME results (total ion count) of two early death cues (3-octanol: grey; 3-octanol: white). Each plot presents results from a specific caste. Results from dissected body parts, head, thorax, abdomen and injured samples were presented together with the living control. Quantities were shown as mean \pm SEM and distribution of samples was demonstrated with black dots. Data were pooled from three *R. flavipes* colonies with 3 replications each.

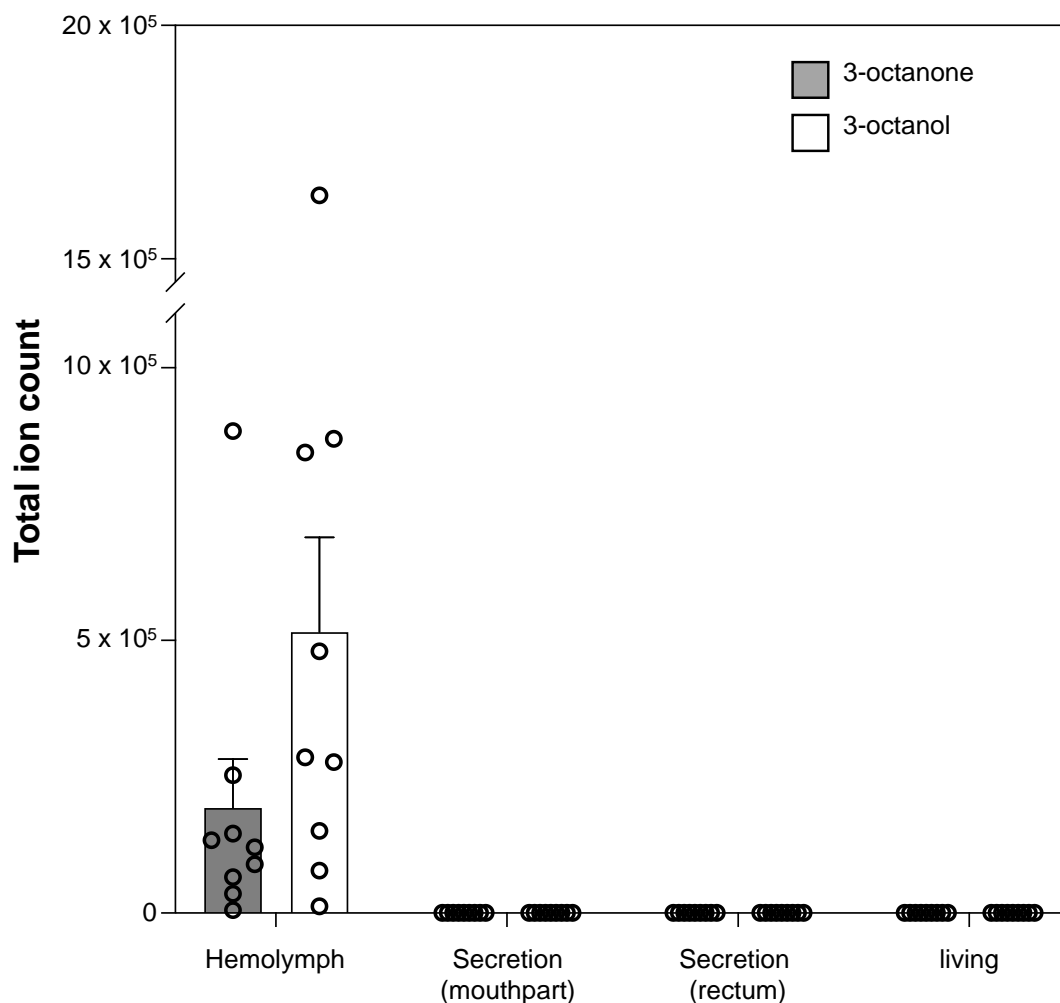


Figure 4.3 Screening potential sources of early death cues from different body fluids in *R. flavipes* workers.

Early death cues were screened using HS-SPME from three different types of body fluids from termite workers. Quantities of 3-octanone (grey) and 3-octanol (white) were presented in the form of total ion count. Samples were prepared from hemolymph, secretions from either mouthpart or rectum, and living workers. Results were presented as mean \pm SEM. Black dots show samples distributed in each group. Three replications were conducted in each of the three colonies.

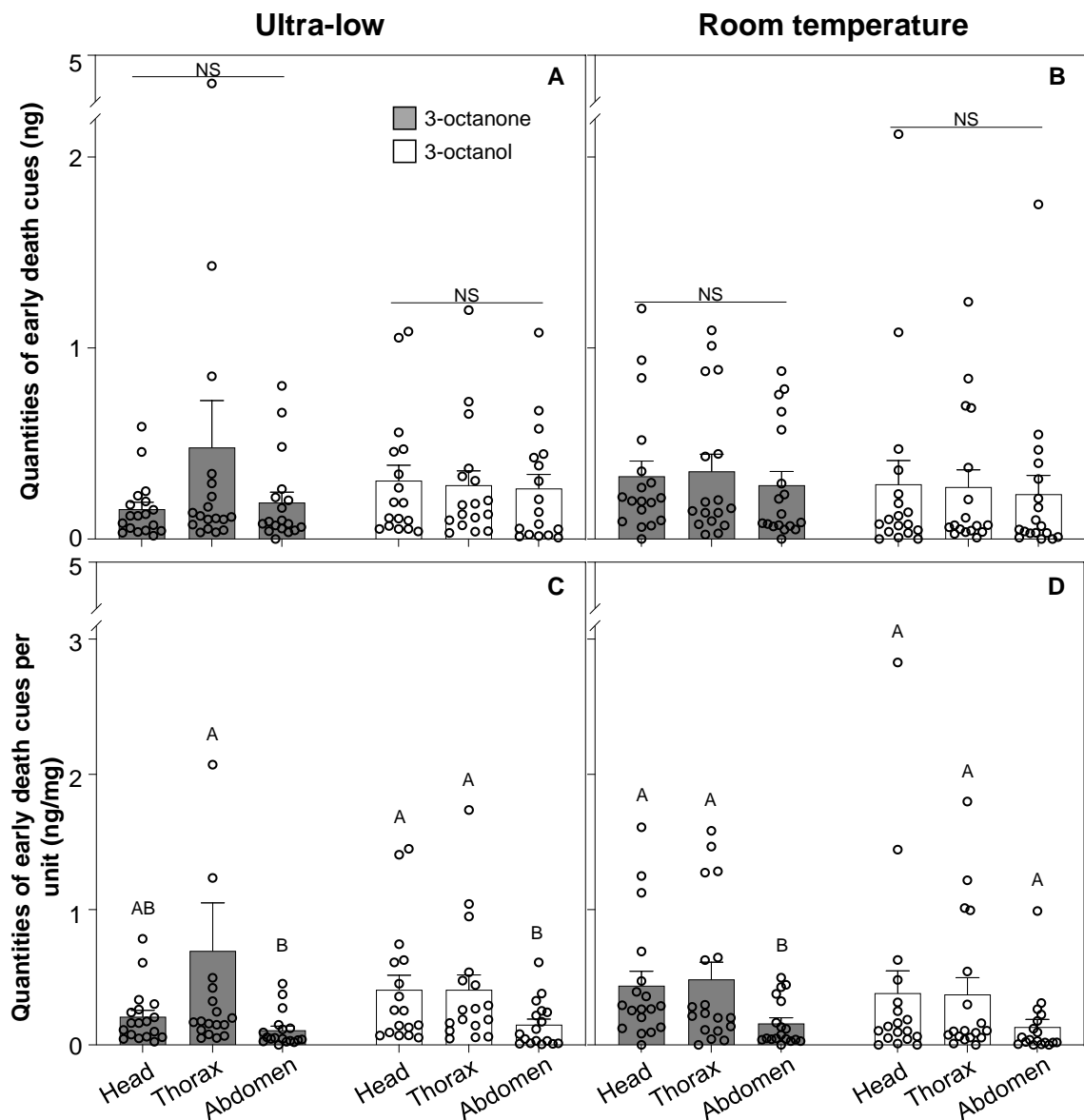


Figure 4.4 Quantities of early death cues in different body parts responding to ultra-low and room temperatures.

Quantities of early death cues (3-octanone as grey and 3-octanol as white) were compared across different body parts (head, thorax and abdomen). Amount detected was calculated either by each body part (upper two figures) or by unit (lower two figures). The right two figures present results from ultra-low treatment and the left ones show room temperature treatment. Data were collected from 3 colonies with 6 replications for each. Significance

across body parts was presented using letters: the same letters above different bars represent no significant difference. NS means “not significant”.

CHAPTER 5. SUMMARY AND PERSPECTIVES

Death as a fundamental phenomenon has been considered to play an important role in evolution, which means all organisms must face death from their conspecifics (Gregory, 2009). How death influences their survival conspecifics in animals is an appealing question but remains largely unknown both proximately and ultimately (Anderson et al., 2018). Social animals, especially eusocial insects, have evolved complicated and delicate corpse management strategies to detect conspecific death and deal with it under influences from frequently changing environment and decomposition status, which imply an adaption to their social living (Sun et al., 2018; Gonçalves and Biro, 2018).

From simply ignoring or avoiding to coordinating different behaviors to a conspecific corpse in eusocial insects, animals evolved diverse death-related behaviors. The evolutionary history of death-related behaviors was studied in Chapter 1 to investigate the questions of 1) which death-related behaviors are more ancestral and which ones are derived and 2) if sociality drives the evolution of death-related behaviors. The reconstruction of ancestral states of death-related behaviors suggested cannibalism on dead conspecifics an ancient and widely employed death-related behavior in animals. Sociality was suggested to be related to the emergence of death-related behaviors and a more complex society might require a larger/more complex death-related behavioral repertoire. Additionally, a consistent rise in interest, as well as a promising integration throughout the currently dispersed field of death-related research, was suggested between research field in vertebrate and invertebrate animals. Topic study through the co-citation analysis might also provide insight for future studies of undertaking behaviors for

termites, such as neuron and genetic basis of undertaking behaviors. Major limitations discussed in this chapter might also reflect the potential shortage in this field, such as small datasets, data need from more animal taxa, especially more “ancient” species and animals missed from previous studies, for example, eusocial mammals. As has been suggested by many researchers in this field (Gonçalves and Biro, 2018), controlled research on animals’ responses toward death is still required for broader taxa for future comparative studies.

Death itself might be more complicated for social insects than other animals. A conspecific corpse might stand for a variety of risks, for example, disease, competitors, and predators. Can animals detect such differences from the information extracted from the dead and deal with it? In Chapters 2 and 3, I’ve combined ethology with chemical ecology trying to address such question from extrinsic (different causes) and intrinsic (different castes) differences, respectively. In nature, animals would die from many extrinsic reasons, whose subsequent dead bodies would be associated with the risks represented by the causes (Hill et al., 2019; Sun et al., 2018). Detecting the chemical signature of the death is the main for eusocial insects to distinguish the dead from the livings (Gonçalves and Biro, 2018). Causes of death might change the chemical nature of corpses along with decomposition, if termites could tell the difference and respond accordingly remains unclear. We predicted that overall termites would use cannibalism as a primary strategy for dealing with death and utilize burial when risks associated with death and decomposition increase. In chapter 2, using both chemical and behavioral analysis approaches, our findings demonstrated that cannibalism predominated during the early stages of decomposition and that biotic causes sped up the utilization of burying

behavior and the accumulation of late death cues. The behavioral discrepancies may be explained by the qualitative and quantitative changes in chemical profiles. Death is a process more than an event. For example, termites would predominantly use grooming for individuals exposed to entomopathogenic fungi, and rapidly switch to cannibalism when the individuals are moribund, whereas burial is rare during this process (Davis et al., 2018). Together with our findings, after being exposed to the fungi, termites are suggested using a coordinated behavioral strategy dealing with pathogenic risks involving groom, cannibalism and burial in accordance to the infection status prior to death, occurrence of death and decomposition after death.

The existence of castes that is reproductively altruistic or sterile is one of the key characteristics of eusociality (Wilson, 1971). Given workers carry out almost all tasks within the colony except reproduction and the importance of the imaginal line's reproductive outputs to the colony, castes from imaginal line and neuter line might represent different types of risks. We hypothesized that based on the postmortem chemical signatures of the corpses from different castes, termites would react to them in various ways. Regardless of the presence or absence of early death signals, the bodies of all castes were retrieved back to the nests and cannibalized when they had been decomposing for less than 64 hours. But after 64 hours, *R. flavipes* employees buried all the bodies on the scene, with the exception of the soldiers. Oleic acid, a late death cue, increased over time in all castes but slowly and at lower concentrations among soldiers. The distinct signaling of the death between the imaginal and neuter developmental pathways may account for the variable release of 3-octanol and 3-octanone between workers/soldiers and nymphs. In both Chapters 2 and 3, being equipped with such an

efficient and selective corpse management strategy would help termites distinguish death with both intrinsic and extrinsic differences and adjust the responses, which could minimize risks associated with them and contribute to subterranean termites' success in their nest-living lives.

Early death cues play an important role in the coordinated behavioral responses between cannibalism and burial of corpses in *R. flavipes*. However, how they are produced and released remains largely unknown in termites. In order to comprehend the early death cues better, the time of biosynthesis and potential sources were investigated. We narrowed the location of their synthesis to head and thorax. Though the exact site of two early death cues still couldn't be confirmed, we suggested further investigations on the distribution of hemolymph during death process may help in looking for the potential sources. Under the assumption of the biosynthesis of early death cues as an enzymatic process, the results also suggested that the synthesis of early death cues might happen before death and retained in the body, and immediately released upon death. Except for as early death cues recruiting workers to cannibalize fresh corpses associated with lower risks, 3-octanol and 3-octanone might also function as a similar recruitment function during termite policing behavior (Sun et al., 2020). Additionally, the antifungal activity has been reported of C8 volatiles, implying a potential individual-level immune function of these two compounds (Hung et al., 2015). The function of these two C8 volatiles during termite alive will be an interesting issue for future study.

Overall, the present dissertation study advances our understanding of evolution of death-related behaviors based on the underlying mechanisms of chemical-based "death recognition" in non-human animals. It would provide insights for further evolutionary

investigations of death-related behaviors and social behaviors related to risk assessment and management in eusocial insects.

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VITA

EDUCATION

- 2020 Graduate Certificate in Applied Statistics, University of Kentucky, USA
- 2012-2015 M.S. in Entomology, China Agricultural University, China
- 2008-2012 B.S. in Entomology, China Agricultural University, China

PROFESSIONAL POSITIONS

- 2015-present Graduate Research Assistant, Department of Entomology, University of Kentucky
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AWARDS AND HONORS

- 2020 Department of Entomology Publication Scholarships, University of Kentucky
- 2015-2019 4-year Doctoral scholarship, China Scholarship Council and University of Kentucky
- 2010, 2013 Social work scholarship, China Agricultural University
- 2010, 2013 Excellent Student Leader Scholarship, China Agricultural University

PUBLICATIONS

- Shi, J.**, Merchant, A., Wang, Q., and Zhou, X. (2022). Signals and cues in death recognition: a quantitative review. *Journal of Plant Protection* 49, 240-249.

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ORAL PRESENTATIONS

2020 **Shi, J.**, Zhang, C., Huang, S., Merchant, A., Sun, Q., Zhou, C., Haynes, K.F., and Zhou, X. Managing corpses from different castes in the eastern subterranean termite, *Reticulitermes flavipes* (Blattodea: Rhinotermitidae). Annual Meeting of the Entomological Society of America, virtual annual meeting.

2019 **Shi, J.**, and Zhou, X. The influence of causes of death on corpse management in *Reticulitermes flavipes*. Annual Meeting of the Entomological Society of America, St Louis, MO.

2019 **Shi, J.**, and Zhou, X. The influence of causes of death on corpse management in *Reticulitermes flavipes*. Annual Forum of the Ohio Valley Entomological Association, Lexington, KY.

2017 **Shi, J.**, and Zhou, X. Impact of different causes of death on the undertaking behaviors in Eastern Subterranean Termite. Annual Forum of the Ohio Valley Entomological Association, Columbus, OH.

POSTER PRESENTATIONS

2022 **Shi, J.**, Sun, Q., Haynes, K.F., and Zhou, X. Evolution of death related behaviors in non-human animals: a systematic review. Departmental retreat of Department of Entomology, University of Kentucky, Lexington, KY.

2022 Sha, L., **Shi, J.**, Yuan, L., and Zhou, X. Isolation and identification of an enzyme in *Manduca sexta* that converts monomeric TIAs into dimeric insecticidal anhydrovinblastine. Departmental retreat of Department of Entomology, University of Kentucky, Lexington, KY.

2019 **Shi, J.**, Sun, Q., Merchant, A., and Zhou, X. The smell of death: A quantitative review. Annual Meeting of the Entomological Society of America, St Louis, MO.