

**A study of foraging behavior and physiological adaptation of
western drywood termite: a framework for development of novel
bandage system**

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PREFACE

This Ph.D. dissertation consists of seven chapters five of which have been published. The major research work for these publications was carried out by BaekYong Choi while the co-authors contributed in the form of overall supervision from experimental designs to manuscript writing. The citations for the published chapters are as under:

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Chapter 4

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CHAPTER 1: General Introduction

Termites are an ancient insect which belongs to the group of insects called Isoptera. There are more than 2,600 different species of termites now recognized. This diversity can be categorized into four distinct groups: subterranean, dampwood, drywood, and arboreal/mound builders (Verma et al., 2009). Termite contributes significantly to most of the world's ecosystem, however, increasing urbanization that involves building in endemic termite habitats and commercial traffic in wood products and infested solid wood materials will likely continue to result in further conflicts between human and termite societies. Consequently, the negative impact of termites is essentially a human perspective. Therefore, the problems associated with termites need to systematically study to provide the feasibility of using less-/no-chemical for control.

1.1 Drywood termites, Kalotermitidae

Drywood termites were first assigned to the family Kalotermitidae in the late 19th century (Froggatt, 1897; Lewis and Forschler, 2014). Currently, 21 genera were recognized within the family Kalotermitidae (Krishna, 2013), however, among those *Cryptotermes brevis* (Walker) and *Incisitermes minor* (Hagen) have considered as the greatest economic important pest of structures since the remaining genera isolate on islands or remote areas of the world.

The biology of the Kalotermitidae is typical of the lower termites and characterized by incomplete metamorphosis development with the usual complement of three primary castes (Parmentier and Roisin, 2003). A reproductive caste of the labor of Kalotermitidae is marked by only a few individuals within population produce king(s) and queen(s) in termite, while a vast majority of no true workers help in raising offspring, and few soldiers protect the colony (Thompson et al., 2000). After the death of the primary reproductive, the worker becomes neotenic replacement

reproductives via a single mount (Fig.1.1). As wood-nesting lower termite species, cast polymorphism influence highly on a high flexible postembryonic development (Korb et al., 2009) and this flexible development are the inherited biological adaptation (Inward et al., 2007).

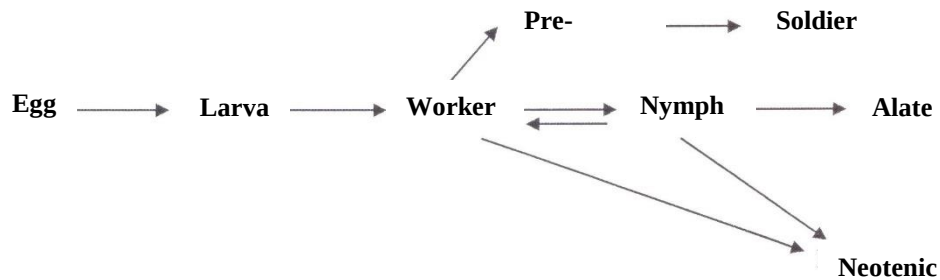


Figure 1.1 Simplified hemimetabolous developmental pathway typical of the Drywood termites
Source: Lewis and Forschler (2014)

The establishment of colonies is established by pairs of winged alates which, after their nuptial flight, seal themselves off in natural checks, cracks, overlapping or adjoining pieces of exposed wood frames in the attic vents, shingle, or foundation vents in urbanized area (Ebeling and Wagner, 1959; Gouge et al., 2009; Scheffrahn and Su, 2014). To establish the mature monogamous colonies, it requires a longer time to mature. Mature monogamous colony consists of up to 400 individual with false workers (Korb, 2007). According to Harvey (1934), there may be one or more supplementary reproductive plus primaries, 70 soldiers, and 1,600 nymphs after ten years. Slow development of colony is mostly due to low-quality food (Korb and Heinze, 2008). Although it required a longer time to mature and smaller size of the population than subterranean termites, prevention of attack by drywood termite is much more challenging due to its cryptic lifestyle and the degree of urbanization the economic impact is high. Based on nesting and feeding habits, termite can be classified into two life type as biological classification (Abe et al., 2000): One-piece type termites and multi-piece type termites. Drywood termites, one-piece type termites, are entirely hidden inside the infested wood, it may be a challenge to detect, though there is an opportunity to

control the infestation by external signs of infestations. Drywood termites live in limited space within their food source and thus they must dispose of waste material from their colonies. As a result, they make small holes in the galleries and occasionally kick out pellets. Pellets tend to accumulate on surfaces located below these holes and are usually the first evidence of drywood termite infestation (Gouge et al., 2009; Lewis and Forschler, 2014). Unlike multi-piece type termites, drywood termites are morphologically and physiologically highly specialized with thicker and more impermeable cuticle (Minnick et al., 1973), and compensation of larger mass of reserve metabolites by reducing body water mass (Choi and Yoshimura, 2016a; Chown et al., 2011; Lockey, 1988; Tucker, 1977; Woodrow et al., 2000). As water relations in drywood termites, they have an ability to obtain water absorbed either directly from the wood on which they feed (oxidative metabolism) (Becker, 2012) or through moisture in their surroundings (Shelton, 2000), extract water from excretion (Brammer and Scheffrahn, 2002), modified rectal pads (Noirot and Noirot-Timothee, 1977), and larger body size (Danks, 2000; Foo et al., 2011; Gibbs et al., 1997). These specific morphological and physiological elements play a vital role to survive exposure to adverse conditions (Cabrera and Rust, 1996).

Drywood termites management is tough for some reasons such as cryptic lifestyle, adaptive biology in xeric conditions, and propagation by transportation of small infested timber. Mainly, mature colonies of drywood termites can survive in a small piece of wood and wood products without free water and can be easily transported from one place to others. The most widespread species belong to the family Kalotermitidae is *C. brevis* and *I. minor*. *C. brevis* is originated from coastal deserts of Peru and Chile, but now they are one of an important pest of structures in many tropical and subtropical regions of the world, except Asia (Gay, 1967; Scheffrahn et al., 2009) (Fig.1.2). There is wide speculation that they might be introduced into other parts of the world by

wooden goods in the 17th century (Bennett, 2012; Scheffrahn and Su, 2014). *I. minor*, whereas, has much smaller world distribution areas than *C. brevis*, but intensively spread in endemic regions, the southwestern United States and isolated regions throughout the North America (Grace et al., 1991; Messenger et al., 2000; Scheffrahn et al., 2001) and other parts of the world including China (Yan et al., 2001) and Japan (Choi and Yoshimura, 2016a; Indrayani et al., 2005; Mori, 1976). Currently, drywood termites, which are a serious pest of timber in buildings and furniture wherever it occurs, appear to have been more widely and most commonly introduced to new world than any other termite species.

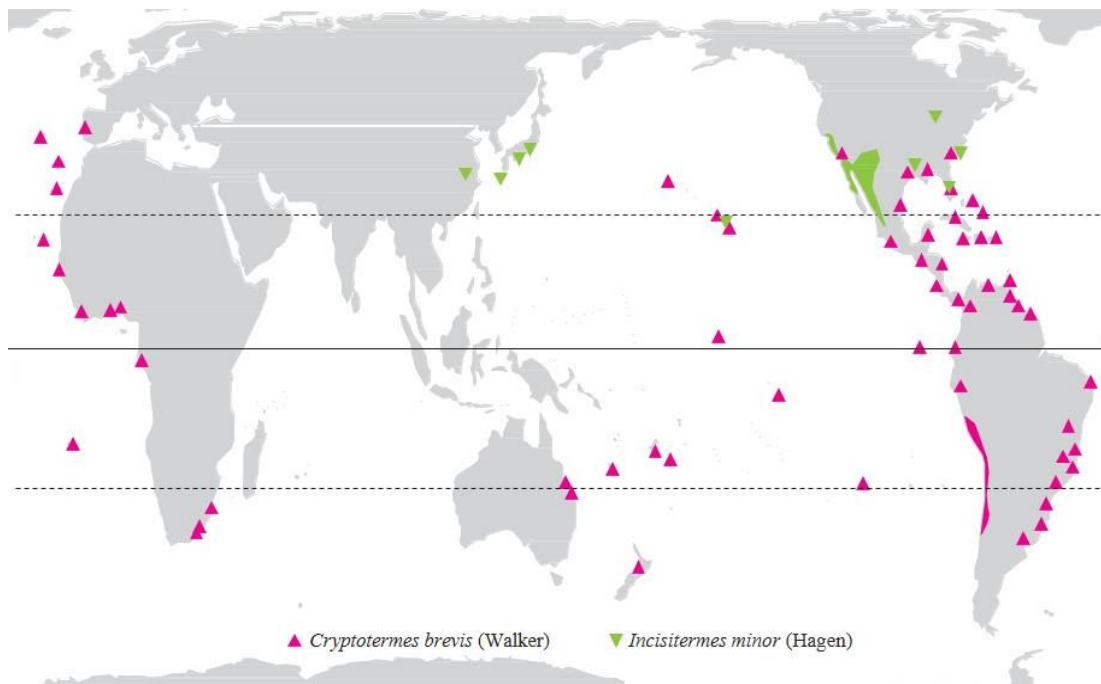


Figure 1.2 World distribution of two most economical important drywood termites, *Cryptotermes brevis* (Walker) and *Incisitermes minor* (Hagen). Source: Annu. Rev. Entomol. 2013

1.2 Invasive drywood termites in Japan

Japan opened its borders to the world in 1868 since that date 433 insect species have been introduced (Mito and Uesugi, 2004). Of these, drywood termites (*Cryptotermes brevis*, *Incisitermes immigrans* and *Incisitermes minor*) represented slightly less than 0.7% of the total of invaded insects Japanese Ministry of the Environment (JME, 2014). Among these invaded drywood termites, *I. minor* is the most common structure-infesting drywood termite in Japan. As mentioned above, the first discovery of the western drywood termite infestation was reported in Tokyo in 1975 (Mori, 1976). There is speculation that *I. minor* was apparently introduced by moving a shipment of Japanese family from Hawaii. After that, it appeared sporadically throughout the country by an increase in international travel and commerce, at the same time, area disturbed by human activity cover a greater area. Latest infestation report from Japan Termite Control Association shows that 24 out of 47 prefecture had been infested by *I. minor* and considered as structure pest in Kansai regions (Fig.1.3). The current distribution of *I. minor* is limited to the coastal area of Japan that indicates international commerce may be a primary source of introduction exotic species.

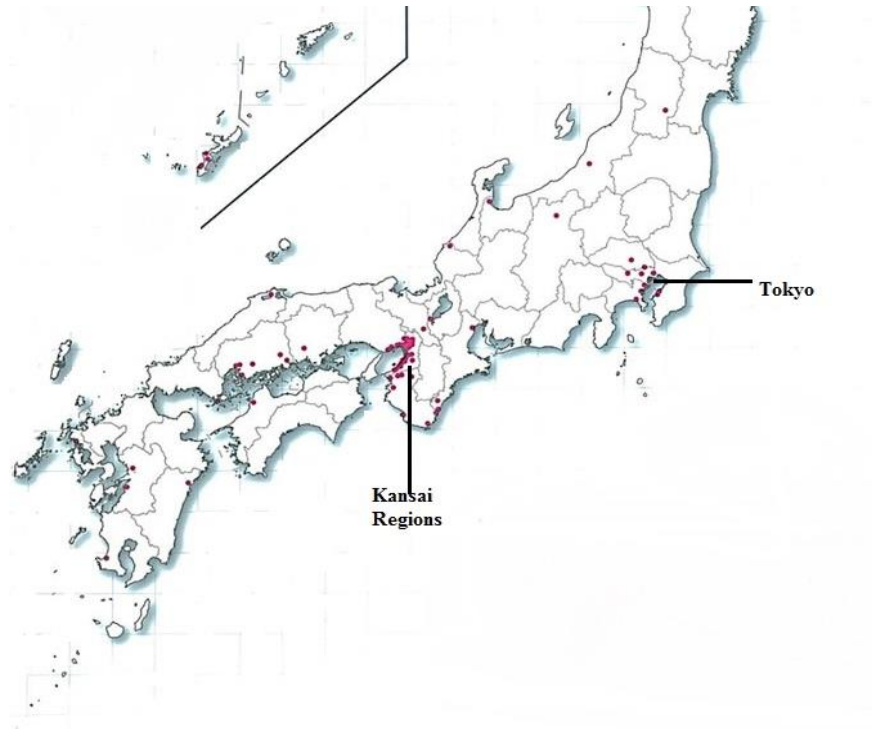


Figure 1.3 Distribution of the western drywood termites, *Incisitermes minor* (Hagen) in Japan

According to Rust and Su (2012), drywood termites account for roughly 20% (ca. US\$ 8.8 billion) of the total estimation of annual termite control and repaired of damage in the world. In comparison with the total economic impact of drywood termite in the world, the production of the total economic impact of termites is much smaller in Japan. At the moment, there is no reliable market information on termite control and repaired of damage by *I. minor* because of relatively short propagation period (less than 40 years) that caused the little economic impact by drywood termites. However, without proper integrated pest management (IPM) it may become a repercussion in next 20-30 years since it also took more than half century to become an economic important structure pest in the United States (Hunt, 1959; Scheffrahn and Su, 2014). As evidenced by the super termite, *Coptotermes formosanus*, exotic termite pest causes devastating damage in the United States.

1.3 Management of invasive drywood termites infestation

The technology associated with drywood termite management has remained unchanged for the past 50 years (Gold 1994) that are categories as a remedial application that intend to kill termites at the time of treatment and preventive application that aim to prevent infestation.

Currently, there are several different treatments for controlling drywood infestations are available depends on intensity, infested location, economic, and environment and working conditions. Most traditional treatment for controlling close or inaccessible locations of infestations of a wooden structure is fumigation with sulfuryl fluoride (Lewis and Haverty, 1996). It is known to be the most efficient to control the termites in the whole structure since an entire structure tightly enveloped with gas-proof tarpaulins, and then a fumigant gas is released into a crack, crevices, and void areas of the structure to kill termite colonies (Fig 1.4a). Whereas it is most costly, disruptive, more laborious, and it does not leave any residual protection after the treatment. Furthermore, the risks of human mortality, undiscovered gas leak and potential lead to explosion and ignition make fumigation structure highly regulated (Su and Scheffrahn, 2000).

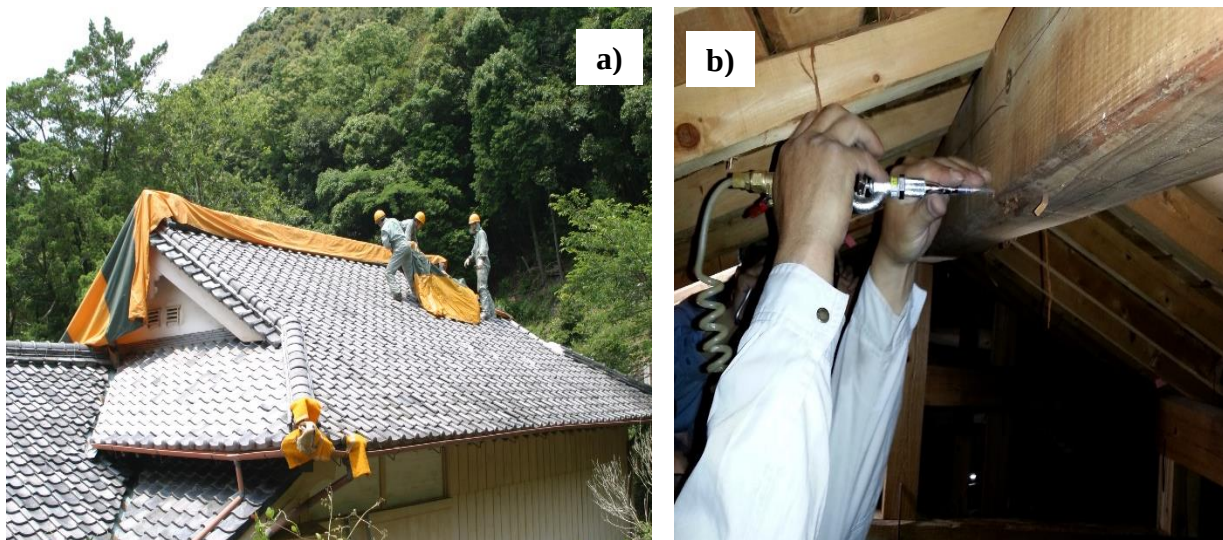


Figure 1.4 a) Fumigation treatment. Photo by Prof. T. Yoshimura b) wood injection spot treatment

Wood injection involving a topical remedy and subsurface pesticide injection have dominated attempts at control of drywood termites (Fig 1.4b). Until 2013, 54 active ingredients formulated into 354 commercial products are listed in the United States (Lewis and Forschler, 2014). The collected data of pesticide uses for topical remedy and wood injection represents at least 70% of the pest management market (Potter, 2004). Similarly, wood injection accounts higher percentage (ca. 90%) of the pest management market in Japan (Based on personal communication with Prof. Yoshimura (2013)). A possible explanation for a high market share of wood injection in Japan is that the simplest, limited areas of infestation, and mostly a competitiveness of a price. The estimation of the cost of localized treatment starts from 20,000 yen (\$165: average exchange rate in 2015) in Japan (Based on personal communication with Dr. Fujimoto (2015)), which is similar to the estimation of localized chemical treatment in the United States, which ranges from \$200 to \$2,000 (Lindell, 1999)

The efficacy of localized application techniques is highly variable based on pesticide elements (active ingredients, the concentration of active ingredients, and formulations) and accurate detection of galleries and chambers. The chemical must be applied to tunnels used by foragers to transmit to other, but if missed them, termites will not terminate. Woodrow et al. (2006) investigated the efficacy of various insecticides in hardwood shipping wooden pallets and reported that numbers of localized injection could not deliver the insecticide which results in low mortality rate (less than 60%).

Insecticide can broadly classified as a natural, inorganic and organic insecticide. Furthermore, it can categorize into two groups, residual and non-residual activity (Ware and Whitacre, 2004). Another way of classifying insecticides is the mode of action (Hassall, 1982). Overall, understanding the mode-of-action is important since it will be toxic to untargeted species in the

environment. At present, there is ten different insecticide for termite are available in Japan (Table 1.1). All insecticide for termite control are classifying as organic based on residual activity, but the mode-of-action is different based on active ingredient.

Although chemical treatment measures are the most widely used and most effectively control the infestation of termite, its toxicity for the environment and human habitat. The public is showing increased interest in non-chemical/less chemical pest control options, which has spurred the development of alternative treatments for drywood termites. At the present, the non-chemical alternative presently marketed for control of drywood termite included excessive heat or cold, microwave, and electrocution (Forbes and Ebeling, 1986; Lewis and Haverty, 1996; Lewis et al., 2000; Rust et al., 1997; Scheffrahn et al., 1997; Scheffrahn et al., 1995). A summary of strength and weakness of commercially available drywood termite treatment options is presented in Table 1.2.

Table 1.1 General information of commercially available insecticide for termite control in Japan¹

Insecticide	Main active ingredient	Mode of action
Aripyreth FL	Bifenthrin	affects the central and peripheral nervous system
Hachikusan FL	Imidacloprid	affects the central and peripheral nervous system
Stealth SC	Chlorfenapyr	Hindering the breath function of insect's mitochondrial
Mikeblock	Dinotefuran	acts as an agonist of insect nicotinic acetylcholine receptors
Mokeblock EC	Dinotefuran	acts as an agonist of insect nicotinic acetylcholine receptors
Optigard ZT	Thiamethoxam	affects the central and peripheral nervous system
Shiroari Mikeblock	Dinotefuran	acts as an agonist of insect nicotinic acetylcholine receptors
Takelock MC50 SUPER	Clothianidin	affects the central and peripheral nervous system
Takelock SP20W	Clothianidin	affects the central and peripheral nervous system
Xylamon trad	Clothianidin	affects the central and peripheral nervous system

¹ (KIHARA, 2013)

Table 1.2 A summary of strength and weakness of commercially available drywood termite treatment options¹

Treatment	Strength	Weakness
Fumigant	•Efficacy confirmed •Whole-/compartmental treatment	• Risk of re-infestation (no chemical residue) • Extremely hazardous • Against the public's demand
Chemical injection and surface treatment	•Efficacy confirmed •Long-term protection	• Detection accuracy is critical to success (No control of undetected termites) • Highly efficient by a.i and concentration • Against the public's demand
Heat	•Efficacy confirmed •Whole-/compartmental treatment	• Detection accuracy is critical to success (No control of undetected termites) • Risk of re-infestation • Fire hazard/ Possible heat damage
Cold	•Compartmental-/localized treatment •Portability •Benign material	• Detection accuracy is critical to success (No control of undetected termites) • Re-infestation • Possible extreme cold temperature damage
Electrocution	•Portability •No chemical used	• Detection accuracy is critical to success (No control of undetected termites) • Limits control (moisture and metal) • Re-infestation • Little damage on structure
Microwaves	•Portability •No chemical used	• Detection accuracy is critical to success (No control of undetected termites) •Limits control (metal) • Re-infestation • Fire hazard/Possible heat damage

¹Sources: Lewis and Rust (2009), Lewis and Forschler (2014), Lewis et al. (2009)

1.4 Development of thermal application

These alternative treatments have developed concerning ectothermic characteristic of drywood termite. Warm-blooded insects' body temperature reflect the temperature of surrounding (Harrison et al., 2012). Based on the idea of natural law, many researchers have been studies on the subject of management of drywood termites (Forbes and Ebeling, 1986; Lewis and Haverty, 1996; Rust et al., 1997) and other pests (Donahaye, 2000; Fields, 1992; Florian, 1990; Gilberg and Brokerhof, 1991; Kells, 2006).

The most representative alternative treatment is the heat treatment. It has been commonly used drywood termite application for last eight decades (Randall and Doody, 1934). Nowadays, this application can use as whole-structure or compartmental treatment (Scheffrahn and Su, 1997) using propane burners, the temperature of air of infested area in the house increases. When the core of timber reaches 54.4°C for 30 minutes, it can generate more than 90 percent of mortality (Quarles, 2006). At the opposite end of the temperature, liquid nitrogen has been tested in both laboratories and fields against drywood termite since the 1980s. Excessive cold is primarily utilized for the compartmental treatment of house (e.g. wall void) by injecting of liquid nitrogen till the temperature drops to a lethal level. Laboratory studies have shown that minus 21.3°C for 5 minutes can generate 100% mortality of drywood termite (Rust et al., 1997). The outcome from these studies allows the pest management company to develop the remedial treatment for drywood termite (Blizzard®) and other urban pests (Cryonite®).

Blizzard system was first introduced in 1985 using liquid nitrogen (− 195.79°C). The mode of action of liquid nitrogen is to drop the temperature of structure timber to below minus 20 degrees Fahrenheit and crystallizes the water in cell membranes to ice, killing the termites. Like other localized treatment, accuracy inspection is critical for the effectiveness of liquid nitrogen treatment.

First, pinpoint the drywood termite colonies by an in-depth inspection, and then drilling small holes in the infested area (wall voids) and pump in the liquid nitrogen. A controlling pest with Blizzard system has its risk. Although nitrogen is not toxic, however when too much nitrogen is released it can act as a simple asphyxiate by displacing the oxygen in the air to levels below that required to support life (Yanisko and Croll, 2012). Hence, the pest control operator must be cautious of ventilating enclosed area. Another system using extreme cold temperature to control urban pests is Cryonite®. The Cryonite® system is introduced to urban pests control market. It generates a powdery carbon dioxide snow (dry ice) through the applicator nozzle. When this dry ice flakes exposure at standard atmosphere, it sublimates which extract the heat energy from the immediate surroundings and ultimate cooling results (Cryonite, 2008). Consequently, the extraction heat energy results in insects' cell crystallize to ice and killing the insects. Both of liquid nitrogen and carbon dioxide are both excellent cryogens, but when the decision to choose one cryogen over another is purely an economic decision and advantage based on the physical properties of the cryogen.

As a remedial application, properly conducted freeze application is effective in a compartment or localized application. Nevertheless, there are questions remain about their effectiveness. All thermal application is effective but leaves no residual protection prompting repeated application. Residual activity of the active ingredient is important to prevent re-infestation because pairs of winged alates will utilize abandoned galleries as first points for new colony formation (Lewis and Forschler, 2014). Therefore, additional research will be needed before these methods are used on a larger scale.

1.5 Two-in-one drywood termite control application

Drywood termite control application relying on a single treatment remains suboptimal since complete protection is not possible in thermal application because of no residual protection. The combination of two or more treatment approaches with different mechanisms can cooperatively prohibit drywood termite elimination and is a promising strategy for effective treatments of termite control with synergistic or combined effects. The concept of two-in-one drywood termite control application evolves into the remedial application using carbon dioxide and preventive application with a liquid insecticide.

1.5.1 Carbon dioxide

Carbon dioxide is colorless and odorless (at a low concentration only) gas. Though, when carbon dioxide is expanded directly to the atmosphere, it becomes a solid carbon dioxide, dry ice. Approximately 50% of carbon dioxide snow (-78.5°C) is generated during expanding, and rest gas produces 50%. However, in most dry ice plant production recovers and recycles into the dry ice process (EIGA, 2008). Once this carbon dioxide snow generates, it can be pressed hydraulically into different shapes and size of dry ice and is utilized by many types of industries, mainly because of its cooling properties. On the contrary, with an elevated pressure (72.9 atm) and temperature (304.25 K), supercritical fluid state, it can dissolve the pesticide (Iversen et al., 2003; Muin et al., 2003).

As mentioned above, both liquid nitrogen and carbon dioxide are excellent cryogens. However, the balance can even be said to be for carbon dioxide. Up to date, carbon dioxide has been used widely in pest management methods, such as fumigation (Athié et al., 1998; Bailey and Banks, 1974; Delate et al., 1995), wood preservation treatment (Acda et al., 2001; Cookson et al., 2009;

Hassan et al., 2001; Iversen et al., 2003; Kotnik et al., 2005; Sahle-Demessie et al., 2003), and freezing treatment (Whitford, 2013).

1.5.2 Non-repellent insecticide: Imidacloprid

Imidacloprid, $C_9H_{10}ClN_5O_2$, is the most widely used insecticide for pest control in agriculture. It is a systemic insecticide which acts as an insect neurotoxin by interfering with the transmission of stimuli in the insect nervous system (Daborn et al., 2001). Its excellent systemic properties that are transported mainly through xylem in growing plants and lasting action make it suitable for soil injection, tree injection, and surface application to the plant skin Suchail et al. (2001).

1.6 Dissertation Objectives

In this dissertation, it was decided to develop the novel two-in-one remedial (provides two treatments at once) bandage application for drywood termite using carbon dioxide (primary function as a remedial application) and non-repellent insecticide (secondary function as a preventive application). The main objective of this dissertation are as follows:

Determine thermal performance of solid carbon dioxide as innovative remedial and preventive drywood termite application: The first aim of this dissertation is desired to investigate and understand which parameters (e.g.: density, sapwood-heartwood, earlywood-latewood, growth rings, cutting direction) affect the thermal performance as the remedial application using carbon dioxide. The thermal performance of solid carbon dioxide is evaluated based on the application of different loading amounts of dry ice to attain the targeted critical thermal minima “CT_{min}” (−21.3°C) of drywood termite. Understanding of cold tolerance parameters (e.g., supercooling point, critical thermal minimum, physiological elements of drywood termite) that help to understand the low temperature performance of drywood termite to optimize the design of the remedial application. As sub-objective, other parameters (e.g., feeding and foraging behavior of

drywood termite, and the toxic threshold of insecticide) are investigated to raise the efficiency of the remedial and preventive application.

Evaluation of the effectiveness of new remedial and preventive drywood termite application: the final objective of this dissertation is to investigate the performance of the novel remedial bandage application on artificially infested timbers and provide a suggestion that will enable a pest management industry to develop more environmentally friendly and efficient drywood termite treatment for the future.

1.7 Dissertation Overview

This dissertation is organized as follows. Chapter 1 introduces the general facts and prevention tips to control drywood termites and motives the need for a close study of drywood termite treatment by solid carbon dioxide. As the first part of the dissertation, understand the foraging behavior and biological adaptations of drywood termites were conducted to provide a framework for improving efforts at detection and control when treatment is necessary. In chapters 2, analyze a one-year laboratory study on foraging behavior and activity of feeding groups of *I. minor* in artificially infested logs of the two most vulnerable structure timber species and demonstrate a more realistic measure of western drywood termite feeding activity using a CT scanning method. In Chapter 3 and 4, we measure the biological variations in an individual group of termite species to compared and identified correlation between the natural cryoprotectants and environmental stress (cold temperature and desiccation). As the second part of the dissertation, the development of two-in-one was conducted with the obtained results from part 1. In Chapter 5, the thermal conductive properties of wood at low temperatures were defined as the cooling rate to the targeted critical thermal minimum (CT_{min}) of drywood termites ($^{\circ}C/min$), the minimum core temperature ($^{\circ}C$), and the duration of the temperature below CT_{min} (min). In Chapter 6, the effectiveness of

designed novel bandage system (remedial and preventive treatment) is presented. Chapter 7, details the overall discussion and conclusions from each chapter and, as well as discuss the further direction in which this study can be carried forth.

CHAPTER 2: Using a computer tomographic method to determine the foraging behavior and feeding activities in groups of the western drywood termite (Isoptera: Kalotermitidae)

This chapter has been submitted to *Holzforschung* and under revision

2.1 Summary

The cryptic lifestyle of drywood termites makes it difficult to study their foraging behavior and detect infestations in hard to reach locations. Therefore, the CT images collected over a one-year observation of drywood termite foraging behavior explained and helped to understand the feeding ecology and, by extension, improved the early detection and monitoring. Likewise, this method contributed to determine more efficient control techniques and follow-up monitoring. According to the CT scanning results obtained, foragers have distinguished foraging patterns based on the physical properties of wood during gallery construction, and foragers primarily excavate along the springwood (radial pattern) towards the outer surface of the wood to establish the primary and satellite chamber, followed by mainly superficial (0.3 — 3.0 cm under the surface) longitudinal expansion, and occasional lateral direction expansion. The development of tunneling is effective by the physical constraints of knots (high lignin content) and upsets (high extractive content). The factors that affect the foraging and feeding activity of drywood termites are highly interrelated with numerous factors that, consequently, influence survival and colony development.

2.2 Introduction

The economic importance of pests heavily depends on international travel and commerce and, at the same time, the degree of urbanization. Invasive western drywood termites, *Incisitermes minor* (Hagen), were first discovered almost 40 years ago in Tokyo, Japan (Mori, 1976) and appeared

sporadically throughout the country. The latest infestation report from the Japan Termite Control Association (JTCA) shows that 24 out of 47 prefectures had been infested by *I. minor* (JTCA, 2015); however, it is not currently classified as a structural pest due to its low economic impact, which is estimated to be approximate US\$ 8 million (Verma et al., 2009). Nevertheless, without proper integrated pest management (IPM), drywood termite may have repercussions in next 10-20 years in Japan. According to several American researchers (Harvey, 1934; Korb and Lenz, 2004; Lenz, 1987), the drywood termite requires a longer time to mature compared to subterranean termites. Moreover, the prevention of damage by the drywood termite is more challenging than the protection against subterranean termite species. As a result, it became an economical important structural pest after more than a half-century from its first discovery in the United States (Hunt, 1959; Scheffrahn and Su, 2014).

The increased importance of western drywood termites as structural pests in Japan is attributable to international travel and commerce, urbanization, and high demand for North American logs. In particular, the latter has resulted in the introduction of non-native timbers into Japan. Haverty (2003) reported a specific information related to risk elements of introduction into non-endemic regions. In this report, he included the potential entry of logs, the potential colonization at ports and the possible spread of infested wood as high-applicable risk criteria.

Wood is the building material for Japan's residential construction, which is the third largest user after the U.S. and Canada (Gaston et al., 2010). Based on the demand of Japanese house consumption, approximately 0.9 million units and 55% of new residential houses were built with wood in 2015 (MLIT, 2015). Due to the intense popularity of Western-style housing, it has translated into a demand for North American wood species. In 2014, approximately 3.1 million cubic metres of North American logs (Douglas-fir: *Pseudotsuga menziesii*, Hemlock: *Tsuga*

heterophylla, and SPF: *Picea glauca*, *Picea mariana*, *Pinus banksiana*) were imported (JLJ, 2015), and these logs were sawn and processed into North American platform frames (2 x 4 and/or 4 x 6) and prefabricated construction (Edgington, 2004; Gaston et al., 2010; Lam et al., 2005). As mentioned by Haverty (2003), the high risk of potential colonization is greatest at ports with similar climate conditions to those of southern California. To demonstrate Haverty's statement, the latest infestation map of Japan shows that most of the infestation occurred near the coastline and was heavily concentrated in Kansai regions (Fig. 1.2). The numerous field case studies of *I. minor* infestation in Kansai regions revealed that North American imported timber species were often found to be damaged by *I. minor* (Indrayani et al., 2005; Indrayani et al., 2004).

The observation of drywood termite foraging behavior explains and helps understand the feeding ecology and, by extension, improves the early detection and monitoring, as well as determines more efficient control techniques and follow-up monitoring. Visual inspection is the traditional procedure for drywood termite infestation (Lewis et al., 2005) and more sophisticated methods, such as acoustic emissions (Fujii et al., 1990; Lewis et al., 2004), microwave (Evans, 2002; Lewis et al., 2000), and infrared heat (Oliver-Villanueva and Abián-Pérez, 2013), are considered standard detection methods. Currently, none of above non-destructive detection/inspection aids is regarded as an ideal detection method for drywood termite pest management because the past and present practices failed to detect the precise locations of infestation, which consequently increased the number of failed treatments. The localized injection treatment after the non-destructive inspection has demonstrated that the average mortality rate ranges from 4-77% (Grace, 2013). Lately, considerable attention has been devoted to studying the cryptic habits of drywood termites using non-destructive detection methods such as computer tomography (CT) to determine the foraging pattern (Himmi et al., 2014; Perna et al., 2008) and social behaviour (Fuchs et al., 2004; Korb and

Foster, 2010; Viana et al., 2013), which offers three-dimensional digital data that can be processed and characterised quickly and precisely.

The deliverable objective of this study was to analyze the foraging behavior of the western drywood termite and demonstrate a more realistic measure of western drywood termite feeding activity using a CT scanning method. The findings presented in our study show the recent completion of a one-year laboratory study on foraging behavior and activity of feeding groups of *I. minor* in artificially infested logs of the two most commonly infested structure timber species (Sitka spruce and Japanese cypress) in Japan.

2.3 Materials and methods

2.3.1 Structural timber species and collection of drywood termite

The structural timber species were selected based on the feeding responses of *I. minor* (a mixture of large larvae, psudergates, and nymphs) to four commonly used structural timber in a preliminary experiment of wood preferences that *I. minor* preferred to attach the density of timber around 450 kg m⁻³ (Choi, unpublished data). Three replications of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Japanese cypress (Hinoki: *Chamaecyparis obtusa*) with dimensions of 85 (H) x 135 (W) x 1000-mm (L) were selected in this study due to their low resistance against *I. minor*. All structural timber species were purchased from the local timber market in 2005 and air-dried in the wood storage room until use. All specimens were made of a combination of heartwood and sapwood, and contained a moisture content below 22%.

Naturally infested structural timbers from residential houses in the Wakayama Prefecture, Japan were moved to the laboratory. Six different pieces of infested structural timbers were selected, and 70 individuals were carefully removed, which consisted mainly of workers and only 2.9% soldiers from an individual piece of wood. The collected individuals were weighed (10 mg each or heavier)

and then maintained in plastic containers with lids. The containers had small pieces of the original timber obtained from the collection before use in the experiment. The collected termites were maintained in a dark and controlled condition room ($28 \pm 2^{\circ}\text{C}$, $>85\%$ RH) for one week to ensure the status of drywood termites. Detailed measurements of the wood specimen and drywood foragers are shown in Table 2.1.

2.3.2 Experimental procedure

To facilitate the introduction of termites, we created a releasing chamber using the base of a petri dish (90-mm diameter) to maintain groups of 68 workers and two soldiers on the surface of timber blocks. A 10-mm hole was created on the base of the petri dish and placed upside down on the surface of a test block with a radial section area. The petri dish and wood specimens were secured with duct tape to prevent the escape of termites. After the construction of samples, pairs of blocks (wood species per colony) were placed on top of stickers (ca. 15-mm) with gaps of 15-mm between each timber block. A total of 70 individual termites on each timber block were maintained in a dark basement at $28 \pm 2^{\circ}\text{C}$, and $> 85\%$ RH for one year. During the initial stage, the number of dead termites in the releasing chamber was recorded daily, and dead termites were removed from the group. After individual termite had been excavated into the entrance hole(s), weekly visual inspections were conducted to examine and record the appearance of kick-out holes.

Table 2.1 Summary of wood properties — mean values and significance of differences based on paired t-test at the 5% significance level

	Japanese cypress	Sitka spruce	Statistic
Forager (mg)	11.0 ± 0.3 ^a	11.2 ± 0.5 ^a	$t = 0.228, P = 0.831$
Density (kg m ⁻³)	487.15 ± 12.33 ^b	437.76 ± 3.29 ^a	$t = 3.869, P = 0.018$
Volume (m ⁻³)	0.01148	0.01148	-
MC (%)	21.1 ± 0.8 ^a	22.0 ± 0.9 ^a	$t = 0.767, P = 0.486$
Heartwood ratio (%)	>75%	50	-
Mortality* (%)	28.1 ± 9.1 ^a	15.9 ± 2.9 ^a	$t = 1.400, P = 0.234$
Recovery number**	44 ± 5 ^a	52 ± 3 ^b	$t = 1.368, P = 0.257$
Total WCV	1,067 ± 302 (6 month) ^a	2,343 ± 330 (6 month) ^b	$t = 2.849, P = 0.046$
(mm ⁻³)	1,867 ± 383 (1 year) ^a	4,322 ± 622 (1 year) ^b	$t = 3.360, P = 0.028$
WCR	0.055 ± 0.004 ^b	0.098 ± 0.008 ^a	$t = 4.997, P = 0.008$
(mg/termite/day)			

The means in the same column followed by different letters are significantly different ($P < 0.05$, paired t-test)

* Mortality during first two weeks of exploratory activity

** Recovery number of termite after one year

2.3.3 X-ray computed tomographic scanning

A semi-annual monitoring was performed with artificially infested structural timbers at Kyushu National Museum. The artificially infested structural timbers were scanned using an X-ray CT (Y.CT Modular320 FPD, YXLON International GmbH, Germany) to obtain the digital data

thickness of 1-mm, 1024 x 1024 pixels, with 3.17 two-dimensional image (2D) slices per mm. The collected digital data were then reconstructed into a three-dimensional image (3D) and a series of virtual cuts using volume graphic software (VGStudio MAX 2.1, Volume Graphic GmbH, Germany). Various densities within timber generated different absorption intensities, which resulted in distinctive opacity and voxel data regions. Delivered 3D images of the voxel data were displayed as discrete values of a density function of the timber. By specifying the opacity of the voxels data, it is possible to generate a visual image of the higher and lower density areas and an image of tunneling chambers in the timber.

2.3.4 Statistical analysis

The wood consumption rate (WCR) was expressed as the weight of wood removed by termites over time (Smythe and Carter, 1970); however, measuring the initial, interim, and final wood weights with test duration was difficult due to the larger size of the wood specimen and the cryptic lifestyle of drywood termites. Therefore, we applied the void space (mm³) of the tunnel galleries obtained from the volume graphic software to estimate the WCR. First, the wood consumption volume (WCV) data, mm³ wood/termite/day, for each sample was calculated using the following equation:

$$WCV = V/T/D$$

where V is the total volume created after 365 days, T is the number of workers removed from the artificially infested wood, and D is the total exposure day (365 days).

With the obtained WCV data, the wood consumption rate (WCR) on an individual basis, mg/termite/day, for each sample was calculated using the following equation:

$$WCR = (W/V)*WCV$$

where W is the total weight of the wood, V is the total volume of the wood, and WCV is the wood consumption volume (mm^{-3} / termite / day).

Differences in the wood consumption volume (WCV) and wood consumption rate (WCR) in Sitka spruce and Japanese cypress were explored using paired t-test (SPSS, 2009).

2.4. Results and discussion

2.4.1 Foraging characteristics

The foraging behavior of a group of individual termites from this study showed that the initial foraging activities were concentrated in the softer areas of the radial surface of both Sitka spruce and Japanese cypress. During the first two weeks of exploratory activity, we observed that workers spontaneously attempted to excavate several areas on the wood surface (gallery construction) to soften and improve its nutritional quality (Fig. 2.1a and 2.1b); especially in construction-grade wood, gallery construction is more important than degradation (Delaplane and Fage, 1990).

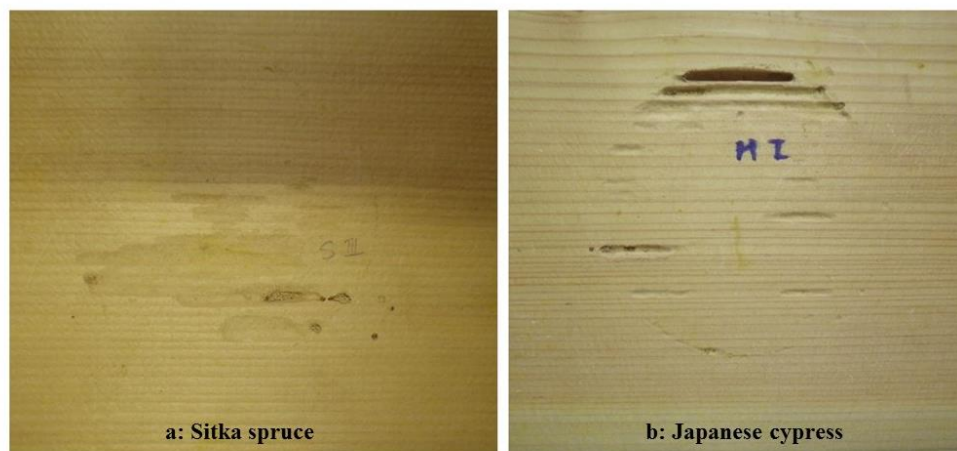


Figure 2.1 Wood surface (gallery construction) during the first two weeks of exploratory activity on a) Sitka spruce (*Picea sitchensis* (Bong.) Carr.) b) Japanese cypress (Hinoki: *Chamaecyparis obtusa*)

During the gallery construction, foragers have distinguished foraging patterns based on the physical properties of the wood. On less dense, wider growth rings with a lower proportion of heartwood in Sitka spruce timbers, the concentration of foraging activities was observed on the wide surface areas that involved several growth rings, whereas foragers on Japanese cypress concentrated their foraging activities on the wider springwood (Fig. 2.2). The factors that affect the wood selection and feeding activities by drywood termites are the nutritional value of timber (Cabrera and Rust, 1999; Grace et al., 2009; Scheffrahn and Rust, 1983), the amounts and chemical nature of wood extracts (Rust and Reiersen, 1977; Scheffrahn and Rust, 1983), and the physical hardness (Evans et al., 2005; Sajap and Sahri, 1983).

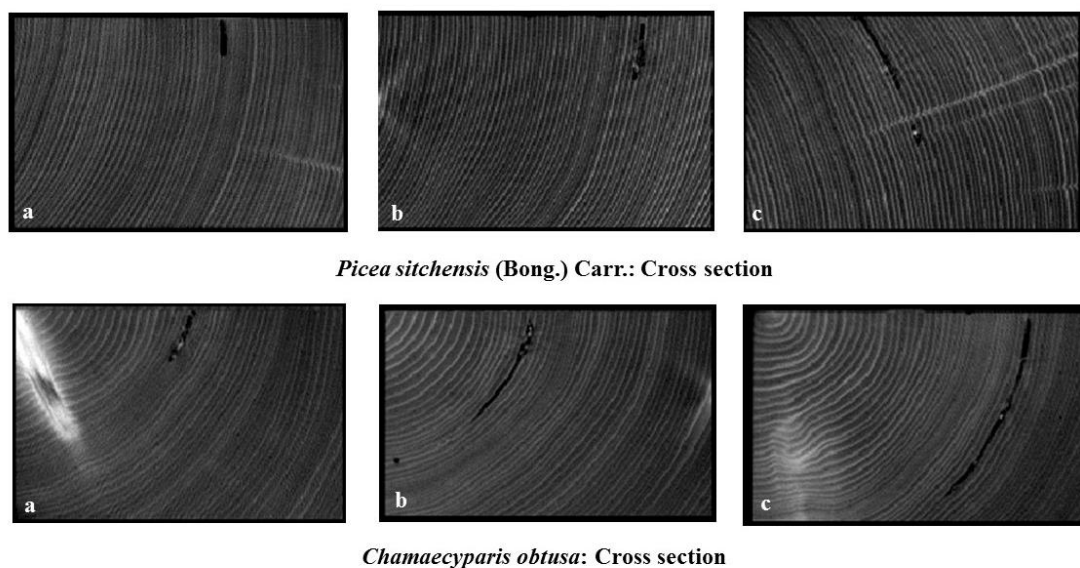


Figure 2.2 2D cross-section image showing the selective excavation of tunnels on the springwood

As described above, the factors that affect the foraging and feeding activities of drywood termites are highly interrelated with numerous factors that consequently influence survival and colony development (McMahan, 1966). Among the most important wood consumption factors are hardness and the presence of toxic substances in the wood (Haverty and Nutting, 1974). Hence, denser materials and the presence of the sesquiterpenoids, such as germacra-1-(10), 5-dien-4 β -ol,

α-cadinol, T-murolol and *α*-terpineol, in Japanese cypress (Kinjo et al., 1988; Morikawa et al., 2014; Ohtani et al., 1997) enhanced the mortality of foragers. Our initial mortality data before the completion of the excavation support the above hypotheses. We observed a significantly higher mortality (mean $\pm$ SE) of foragers on Japanese cypress ($28.1 \pm 9.1\%$) than on Sitka spruce ($15.9 \pm 2.9\%$) during the early excavation period (Table 2.1). Within the survived foragers, drywood foragers require between 12 and 48 h to establish a burrow based on the condition of the wood, and they complete the excavation after 14 days on both wood species.

Another foraging behavior is that foragers primarily excavate along the springwood (radial axis) towards the outer surface of the wood to establish the primary and satellite chambers, followed by a mainly longitudinal expansion, and occasionally lateral direction expansion. From the observations of the nest gallery system (Fig. 2.2 and Fig. 2.3), it is clear that the foragers made their foraging decision without making significant galleries in the wood. The primary chamber was constructed approximately > 10 -mm beneath the surface of the wood. Himmi et al. (2016) estimated that the main chamber had a sufficient space, which varies from 64 to 133 mm³, to aggregate 12 to 20 termites. The tunnel excavation along the radial axis was used to increase the number of chambers, which affected the void space of the wood, whereas the longitudinal tunnels excavation affected the length of the galleries. The irregular longitudinal expansion is the common foraging behavior of the drywood termite (Grace et al., 2009). Regarding the point of decision of longitudinal expansion, we discovered that when the excavation galleries closely reached the opposite surface of the wood, the foragers made their foraging decision rapidly and left a thin layer of barrier between the galleries and outside environment (Fig. 2.3).

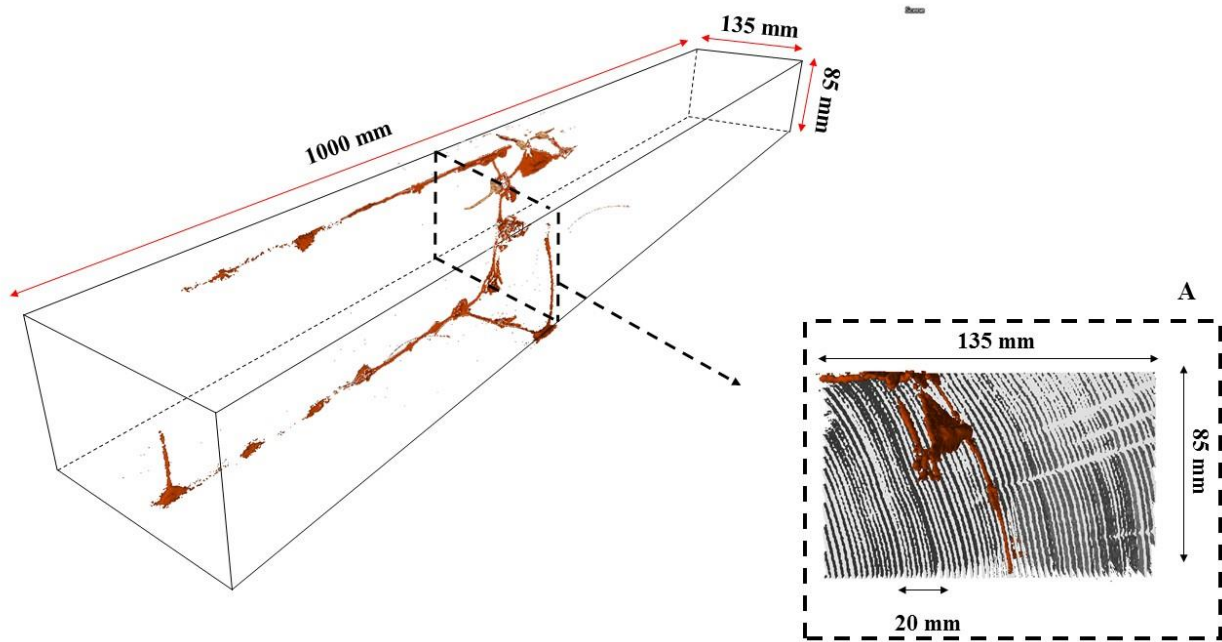


Figure 2.3 3D CT image of the gallery system of Sitka spruce timber C after one year. A) 2D cross-section image showing the selective excavation of the primary chamber and tunnels on the springwood

Consequently, the outer shell of infested wood can be easily broken and can reveal the underlying galleries and large amounts of pellets during inspection (Cabrera and Scheffrahn, 2005). This odd phenomenon is explained in the works of the Evans research team (Evans et al., 2007; Evans et al., 2005) and Inta et al. (2007). The vibratory characteristics of materials are strongly dependent on its properties (Thomson, 1993). During foraging, the termite behavior has evolved to cope with vibration signals to determine the thickness of the wood. Observation of the foraging behavior of the drywood termite during the one year of experimentation support this hypothesis. As a side result, drywood foragers constructed most of the superficial tunnels (0.3–3.0 cm under the surface) with a dimension that ranged between 2.0-mm and 4.5-mm.

2.4.2 Wood consumption rate

Wood density considers the physical factors that influence wood consumption by drywood termites (Rust and Reiersen, 1977) and other termite species. There was a significant correlation between increasing density of the wood and decreasing wood consumption rate by the foragers. Our result shows that the mean ($\pm$ SE) of the total void space of chambers and concealed paths in all three replicates of Sitka spruce was $2,343 \pm 330 \text{ mm}^3$ ($0.020 \pm 0.003\%$ of total wood volume) and this value was $1,067 \pm 302 \text{ mm}^3$ ($0.009 \pm 0.003\%$ of total wood volume) for Japanese cypress over the half-year observation period. These values were $4,322 \pm 622 \text{ mm}^3$ ($0.038 \pm 0.005\%$ of total wood volume) and $1,867 \pm 383 \text{ mm}^3$ ($0.016 \pm 0.003\%$ of total wood volume), respectively, over the one-year observation period (Table 2.1). On an individual basis, each termite consumed approximately 0.098 mg of Sitka spruce each day, while each termite consumed approximately 0.055 mg of Japanese cypress each day (*t*-test for paired samples; $t = 4.997$, $df = 4$, $P = 0.008$). The drywood termite wood consumption appears to be correlated with wood density. However, the significant difference of the total void spaces between the two wood species indicates that the physical factor might not be the sole factor providing resistance to drywood termite. Collected 2D digital data of the nest-gallery development of drywood foragers on Japanese cypress revealed that the development of tunneling is affected by the physical constraints of knots and upsets (Fig. 2.2). The tunneling strategy in response to the physical constraints of knots reported by Himmi et al. (2016). When termite foragers encountered knots, the direction of excavation was changed to the less dense surrounding of knots because below and above knots are high in reaction wood, which is composed of high lignin content (Fengel and Wegener, 1983). Several studies (Ragon et al., 2008; Rust and Reiersen, 1977; Sajap and Sahri, 1983) suggest that the high resistance of termites can be explained by the chemical factors since there is a close relationship between the percentage

of extractives and the natural durability of the wood. Within chemical factors, the amount and chemistry of lignin may influence the termite resistance of the wood (Judd and Corbin, 2009; Ohmura et al., 1999; Wong et al., 2005). Another natural defect in wood (i.e., upsets) may also influence the wood consumption rate of drywood termites. The upsets are mainly due to exposure of the tree, in its young age, too fast blowing the wind, which causes the wood fibers to compress. The occurrence of mechanical stresses induce the trees to produce and deposit protecting compounds, which are extractives (Pereira et al., 2003). From this study, it was assumed that the termite resistance of Japanese cypress comes from the poor nutritional quality of the substrate.

2.4.3 Conclusions

The aim of this study was to analyze the foraging behavior of western drywood termite and demonstrate a more realistic measure of western drywood termite foraging behavior using the CT scanning method. The CT images collected over a one-year observation showed that the galleries of *I. minor* consist of irregular excavations throughout the less dense part of the feeding site, which indicates that a cost-benefit relationship may affect the foraging behavior and reveals that drywood termite resistance appears to be correlated with several factors. One factor is the wood density that affects the chewing ability of the termite. However, more importantly, the presence of extractive and high lignin content, which makes the wood toxic to termite. According to the results of foraging behavior, the drywood foragers construct a radial pattern of principal and superficial longitudinal and lateral tunnels (1-3 cm under the surface) throughout the wood.

To summarize, the presented CT scanning method with continuous research that provides precise anatomical information could help to disentangle the specific physical factors of wood that influence nest structure, while also provides accurate information about the underlying behavioral mechanisms of the drywood termite. However, to deploy this technique on a commercial scale by

the pest management industry, the CT scanning techniques should be refined regarding a portable application to use as standard detection device by the pest management industry.

CHAPTER 3: The role of physiological elements of the western drywood termite (Isoptera: Kalotermitidae) against low temperature stress

This chapter has been submitted to Environmental Entomology and under revision.

3.1 Summary

Colonies of the western drywood termites, *Incisitermes minor* (Hagen), cannot retreat or escape when unfavorable conditions are encountered, compared with subterranean termites. Therefore, a reasonable assumption is that the desiccation and cold hardiness tolerance of *I. minor*, are likely greater than those of subterranean termite species. In this study, we examined ecological adaptations in physiological elements such as water relations, metabolites, and cuticular structure to determine how *I. minor* withstands low temperature stress. The supercooling points (SCP) varied between -10.5°C and -14.5°C, whereas the less lethal temperature (LLT) ranged from -13.9°C to -14.8°C for LLT₅₀ and was below -22.0°C for LLT₉₅. Cold tolerance was influenced by some physiological elements that included low body water content and hardened structure of the cuticle. Although trehalose is a cryoprotectant in termites, the indication was that trehalose did not function as a natural cryoprotectant in *I. minor*.

3.2 Introduction

In contrast to endothermic animals, the body temperature of termites is regulated by the temperature of the environment (Harrison et al., 2012), and termites have evolved a diversity of competitive biological adaptations to ensure survival under sub-optimal temperatures. Numerous comparative studies show that termites effectively manage body temperature with behavioral adaptations such as aggregation and burrowing activity (Hu and Appel, 2004; Lacey et al., 2010)

and physiological adaptations that affect rates of body water loss and metabolism and control of water content in the labial gland (Gautam and Henderson, 2014; Sponsler and Appel, 1990). Together, these adaptations increase the chances of survival.

For subterranean termite species, behavioral avoidance of low temperatures is more important than increases in physiological cold tolerance (Cabrera and Kamble, 2001; Hu and Song, 2007; Strack and Myles, 1997), and habitat exploration is a classic example of a subterranean termite behavioral trait. *Reticulitermes* spp. detect seasonal changes in soil and relocate to deeper ground (Clarke et al., 2013) or to heated structures in an urban habitat (Myles and Grace, 1991) in which the temperature is buffered from the climate. However, in contrast to subterranean termites, the refugia for drywood termites to relocate to are limited when ambient conditions become unfavorable since the galleries of drywood termites are within the air-dried timbers in hot and dry areas of buildings with no contact to the soil (Cabrera and Rust, 2000; Su and Scheffrahn, 1990). Because drywood termites must withstand extreme fluctuations in temperatures and xeric habitats, several competitive biological adaptations evolved for such adverse environmental conditions. The tolerance of drywood termites for unfavorable conditions is usually linked to physiological adaptations that likely evolved to regulate body water content (Steward, 1982; Steward, 1981). Although many studies find that insects synthesize cryoprotectants such as polyols, glycerol (Lee et al., 1987; Sømme, 1982) and cryoprotectant metabolites, low-molecular-mass carbohydrates, trehalose “non-reducing sugar” (Fuller, 2004; Rudolph and Crowe, 1985; Storey and Storey, 1991; Thompson, 2003), glucose “reducing sugar” (Costanzo et al., 1993; Storey and Storey, 1985), and fatty acid ligands (Overgaard et al., 2007; Roy et al., 1991) are cryoprotectants in many species of insects that stabilize insect membranes during hypertonic environment exposure. Most recently, research on the physiological adaptations of other one-piece-type (use the same piece of wood as

their shelter and food) termite species, *Porotermes adamsoni* Froggatt and *Stolotermes victoriensis* Hill, revealed that the cold tolerance of these species is enhanced with the synthesis of trehalose and unsaturated lipids (Lacey et al., 2010). The wide range of lipids found in the whole bodies of insects include among other compounds, significant amounts of lipids secreted in cuticular waxes (Gilby, 1965). The cuticular lipids are composed primarily of n-alkanes, alkenes, and methyl-branched saturated and unsaturated hydrocarbons. These cuticular hydrocarbons increase resistance to desiccation (Collins, 1969; Gibbs et al., 2003), increase cold tolerance (Arrese and Soulages, 2010; Sinclair et al., 2003b; Woodrow et al., 2000) and act as chemical messengers (Richard and Hunt, 2013). Although recent studies on Isoptera show that physiological adaptations can increase the chances of survival under unfavorable conditions, research on the correlations between physiological properties and the cold tolerance of termites continues to be lacking.

The use of extremely low temperature to control subterranean termite pests has been investigated since the 1980s (Cabrera and Kamble, 2001, 2004; Cabrera and Rust, 2000; Clarke et al., 2013; Davis and Kamble, 1994; Lewis and Haverty, 1996; Sponsler and Appel, 1991). Most of these studies explored the supercooling points (SCP) and less lethal temperatures (LLT) of the termite *Reticulitermes flavipes* (Kollar). Based on the results of these studies, *R. flavipes* survives a temperature below -3°C, and the LLT increases and decreases based on the season. In a pioneering experiment for a low thermal method to control drywood termites, liquid nitrogen was applied into wall voids to create temperatures below the LLT, and *I. minor* died when exposed to a temperature below -18.5°C (Rust et al., 1997). Compared with subterranean termites, the LLT of *I. minor* is less, but to our knowledge, no published reports are available on the biological variables that affect the low temperature tolerances of the three structural termite pests examined in this study.

In the present study, we examined the correlations between the genetic adaptations of physiological elements and the cold tolerance of the three most widely distributed pest species in Japan: *Reticulitermes speratus* (Kolbe), *Coptotermes formosanus* Shiraki, and *Incisitermes minor* (Hagen). First, an ultra-cooling freezer was used to estimate and compare the SCPs and the LLTs of the three termite species. Second, the biological variations in an individual group of each termite species were compared to identify correlations between the natural cryoprotectants and the cold tolerance of these three structural pests in Japan.

3.3 Materials and methods

3.3.1 Collection of termites

Three colonies were collected for each of three termite species in this study. The colonies of *R. speratus* were collected from Japanese black pine, *Pinus thunbergii* (Parl.), on the Kyoto University-Uji campus, Japan. Three colonies of *C. formosanus* were from the colonies maintained at the Deterioration Organisms Laboratory (DOL) of the Research Institute for Sustainable Humanosphere (RISH), Kyoto University. The three colonies of *I. minor* were collected from naturally infested structural timbers from residential houses in Wakayama Prefecture, Japan. Before determination of the SCP and LLT and water content, the collected individuals were weighed and maintained in plastic containers with lids that contained original pieces of timber obtained from the collection site. Other sets of freshly collected termites were weighed and then immediately placed in an incubator at -20°C until analyses for trehalose and cuticular lipid contents.

3.3.2 Thermal response of individuals

Thermal response experiments (i.e., determination of SCP and the LLT) were conducted on 54 total replicates. To determine the SCPs, we used contact thermocouple thermometry with a slight modification, as described by Hanson and Venette (2013). A 24-gauge copper-constantan

thermocouple wire was formed into a spiral and placed in a 2 mL syringe tube; a gelatin capsule (110 x 80 x 200 mm) that contained an individual worker was placed on top of the spiral thermocouple wire. Instantaneous readings of data were collected to a computer via a thermocouple interface and SoftThermo E830 software (Technol Seven, Tokyo, Japan). The SCP measuring devices were cooled to -20.0°C at a constant rate of -1.0°C per minute inside an incubator (Eyela KCL-2000A, Tokyo, Japan). The SCP was recorded from the temperature plots as the lowest temperature reached before the release of latent heat of fusion.

For analyses of the less lethal temperatures (LTT₅₀ and LLT₉₅), ten termites per replication were placed in a glass Petri dish (60 mm x 15mm) containing a Whatman™ no. 2 filter paper (Ø 5.5 cm). Termites were placed into the incubator at a natural temperature (control), and then the temperature of the Petri dishes was progressively lowered at -1°C min⁻¹ to the test temperature at which it remained for 30 min. After the 30 min period of low temperature exposure, the indices of mortality were determined by separating the dead and live individuals. Normal termites showed free activity and were able to flip the body back onto its feet within 10 min and dead termite showed no response to touch.

3.3.3 Body water and lipid assessment

A replication consisted of 15 termites (a mixture of large larvae, psudergates, and nymphs), and 18 replications (two replicates/colony/species) were used to determine total body water (%TBW), cumulative %TBW loss, and cuticular permeability (CP). The CP was determined during 24 h of desiccation (RH below 2%) in a chamber containing 1.5 kg of anhydrous CaSO₄, which was previously dried at 100°C for 48 h. The CP value was calculated based on the period of maximal water loss (first 2 h), which was applied to the following two formulas:

Meeh's formula (1879): $S = 12M^{2/3}$, where S = surface area (cm²) and M = initial mass (g); and

Haagsma's formula (1996): $y = 0.0886 + (26.85 \cdot x) - 214.21 \cdot x^2$, where y = surface area (cm^2) and x = initial mass (g).

The CP was defined as water loss [initial mass – mass after first 2 h of the experiment] ($\mu\text{g H}_2\text{O}$) per unit surface area (cm^2) per unit time (h^{-1}) per unit saturation deficit (mm Hg^{-1}). For the saturation deficit, 26.95 mm Hg was used because the tests occurred at only one temperature (28°C) and RH ($< 2\%$).

Immersion of a trisectioned body with 2.0 mL of chloroform for 48 h was performed to estimate the total body lipid content of dried termites. After extraction, the exoskeletons in the extraction solution were removed by filtration through an Omnipore membrane filter ($0.2 \mu\text{m}$ JG-type; Darmstadt, Germany). To obtain the dry mass of the exoskeletons without total body lipids, the extract of total body lipids was dried at 60°C for 48 h to obtain the dry mass. Mass of total lipids was applied to the following formula:

Total body mass (%) = $([\text{dry mass before extraction} - \text{dry mass after extraction}]/\text{dry mass before extraction}) \times 100$

3.3.4 Analysis of natural cryoprotectant, trehalose

For the analysis of carbohydrate metabolites, six replicates of frozen termites (detailed information is provided in Table 3.1) were prepared for each termite species. For each replicate, termites were dried at 100°C for 10 min followed by additional drying at 60°C for 48 h followed by homogenization. A total of 100 mg of dried termites with 3.0 mL of ionized water was placed in a Potter-Elvehjem ice bath homogenizer (Sigma-Aldrich, St. Louis, MO, USA). The homogenates were centrifuged at 15,000 g for 10 min (Kubota 3500; Kubota, Tokyo, Japan). One milliliter of supernatant and 1.0 mL of 1 mol HCl were mixed and sealed and heated for 60 min in

a thermal bath (100°C). After cooling down, the mixture was mixed with 1.0 mL of 1 mol NaOH for neutralization. Neutralized samples were stored in a refrigerator until analysis. Trehalose concentration in the hydrolysates of the termite homogenates was determined based on the methods described by Nelson (1944) and Somogyi (1952).

3.3.5 Cuticular lipids (hydrocarbons) analysis

To determine the lipid composition of the cuticle of frozen individuals (45 workers for *I. minor*, 270 for *C. formosanus*, and 300 for *R. speratus*), termites were dried in a vacuum over desiccating silica gel for two weeks. After obtaining dry mass, individuals were immersed with 10 mL of *n*-hexane (Nacalai Tesque, suitable for HPLC analysis, Kyoto, Japan) for 10 min. The suspension was separated from other components by pipetting through 4 cm of activated BioSil-A (silica gen, Nacalai Tesque, 230-400 mesh, Kyoto, Japan) in Pasteur pipet minicolumns (5 mm). Filtered extracted substances were evaporated to dryness under nitrogen and redissolved in 10 µl of *n*-hexane for gas chromatography-mass spectrometry (GC-MS) analyses. Analysis of cuticular hydrocarbons was performed on GC-MS (QR5000; Shimadzu, Kyoto, Japan) using a wide-bore capillary column (30 m x 0.25 mm ID, HP-1, Santa Clara, CA), and operated in the split mode (33:1). The temperature was increased 3°C/min from starting temperature of 200°C to 300°C with a final holding time of 12 min (Haverty et al., 1988). Compared with external *n*-alkane standards, unknown hydrocarbons were classified by carbon number according to their retention times (Restek, Bellefonte, PA). The quantities of total hydrocarbons was automatically calculated using the proportion of each peak and summing the areas.

After extraction of cuticular hydrocarbons, exoskeletons were dried at 60°C for 48 h to obtain dry mass. After extraction and drying, the percentage of cuticular lipids was estimated as $[(\text{dry mass before extraction} - \text{dry mass after extraction}) / \text{dry mass before extraction}] \times 100$.

3.3.6 Statistical analysis

For determination of the temperatures that induced 50% and 95% mortality of termites, a probit analysis was conducted to correlate the change in mortality with the exposure time (SPSS, 2009). Before analysis of covariance (ANCOVA), all percentage data were arcsine square root transformed. ANCOVA was used to compare the trehalose content and cuticular lipids (dependent variables) data for termite species (independent variable) over dry mass (covariate) and the bioassay and total body water and total lipids content (independent variables) over wet mass (covariate). The data were then compared and separated by Tukey's post-hoc tests ($P \leq 0.05$) and general linear models with termite species to determine possible differences among termite species (SPSS, 2009).

3.4 Result

3.4.1 Supercooling points of three structural pests

As illustrated in Fig. 3.1, body mass was one predictor of the supercooling point (SCP) of the three termite species. We recorded significantly less SCPs for the drywood termite than for the subterranean termites without the covariate (as body mass) ($F_{(2,51)} = 214.072$, $P < 0.0001$) and with the covariate ($F_{(2,48)} = 3.539$, $P = 0.037$). The SCPs of *I. minor* varied between -10.5°C and -14.5°C (-12.6 ± 0.3 ; mean $\pm$ SE), whereas the SCPs of the two subterranean termites ranged from -7.7°C to -11.2°C (-9.3 ± 0.2) for *C. formosanus* and from -4.6°C to -7.4°C (-5.7 ± 0.2) for *R. speratus* ($t_{(34)} = -11.592$; $P < 0.0001$).

The release of latent heat of fusion increased the body temperature of subterranean termites slightly by approximately 0.1°C to 0.3°C and that of drywood termites by approximately 0.4°C to 0.8°C. The increase in temperature was sustained by *I. minor* 2.2-fold longer than that of *C.*

formosanus and 3.4-fold longer than that of *R. speratus*; however, the difference between the subterranean termite species was not significant ($F_{(1,32)}=0.727$; $P = 0.400$; Table 4.1).

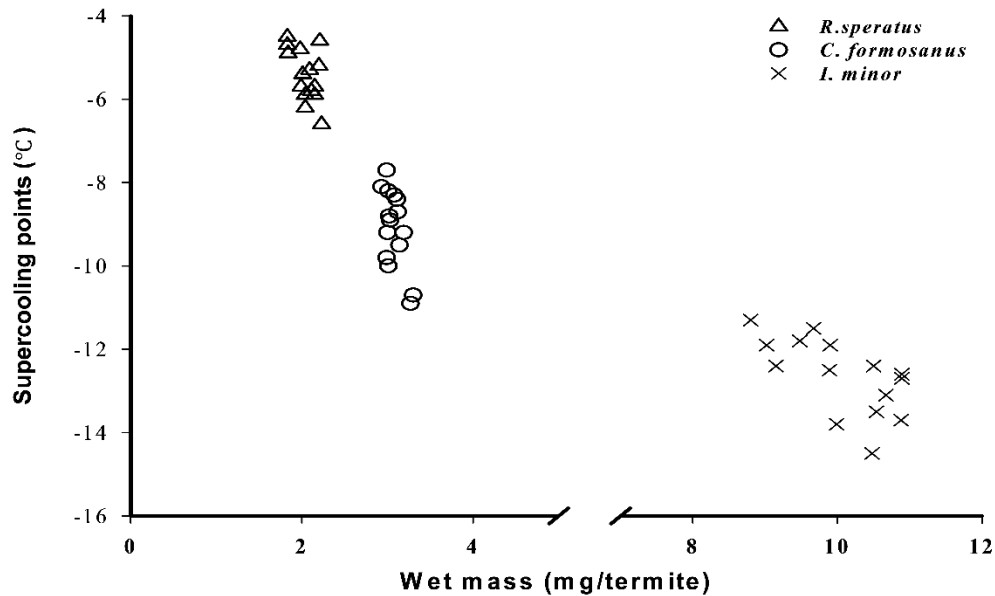


Figure 3.1 Scatterplot showing the relationship between wet mass and SCPs of three termite species

3.4.2 Lethal low temperatures (LLT₅₀ and LLT₉₅)

Table 3.1 shows the responses of the two subterranean termite species and the species of drywood termite following 30 min exposure to different temperatures that ranged from -4.0°C to -22.0°C. When all termite species were exposed to low lethal temperatures, all individuals entered into a chill coma; however, as long as the temperature did not dip below their lethal temperature level, all individuals revived rapidly after rewarming. Individuals of *R. speratus* exposed to -4.0°C and -5.0°C recovered from a chill coma after rewarming, although frozen individuals were observed as the temperature (that exposed termites experienced) decreased. Mortality was first recorded (ca/. 8.0%) at -6°C. With extended temperature exposure at -8.0°C, we observed a sudden surge in

mortality (ca. 68.0%). The calculated low lethal temperatures (LLT₅₀ and LLT₉₅) of *R. speratus* workers were $-6.3 \pm 0.1^{\circ}\text{C}$ and $-9.8 \pm 0.2^{\circ}\text{C}$, respectively. An independent-samples t-test revealed that the LLTs of *C. formosanus* were significantly less than those of *R. speratus*. The LLT₅₀ of *C. formosanus* was between -9.2°C and -11.4°C , and the LLT₉₅ was below -11.4°C ($t_{(34)} = -14.439$, $P < 0.0001$ for LLT₅₀; $t_{(34)} = -12.061$, $P < 0.0001$ for LLT₉₅). A significant species by body mass interaction was detected for the LLTs ($F_{(3,50)} = 273.011$, $P < 0.0001$ for LLT₅₀; $F_{(3,50)} = 846.944$, $P < 0.0001$ for LLT₉₅) of *I. minor* workers, which varied between -13.9°C and -14.8°C for the LLT₅₀ and were below -22.0°C for the LLT₉₅.

Table 3.1 Mean wet and dry mass, water relations, CP, and total lipid composition (mean $\pm$ SE) of three termite species

Elements	<i>I. minor</i>	<i>C. formosanus</i>	<i>R. speratus</i>
Wet body mass (mg)	9.96 $\pm$ 0.17 ^a	3.07 $\pm$ 0.02 ^b	2.08 $\pm$ 0.03 ^c
Dry body mass (mg)	2.78 $\pm$ 0.04 ^a	0.64 $\pm$ 0.01 ^b	0.41 $\pm$ 0.01 ^c
TBW content (%) [*]	72.0 $\pm$ 0.6 ^c	78.6 $\pm$ 0.5 ^b	80.1 $\pm$ 0.3 ^a
Low RH — CP ^{**}	(M) 2.11 $\pm$ 0.04 ^b	(M) 28.67 $\pm$ 0.67 ^a	(M) 31.26 $\pm$ 0.67 ^a
($\mu\text{gH}_2\text{O cm}^{-2}\text{h}^{-1}\text{ mmHg}^{-1}$)	(H) 3.36 $\pm$ 0.09 ^b	(H) 42.13 $\pm$ 0.86 ^a	(H) 42.04 $\pm$ 0.90 ^a
Body lipid (% of dry weight)	28.45 $\pm$ 0.53 ^a	17.32 $\pm$ 0.42 ^c	19.93 $\pm$ 0.49 ^b
Cuticular lipid (% of dry weight)	17.97 $\pm$ 0.41 ^a	9.10 $\pm$ 0.21 ^b	7.28 $\pm$ 0.25 ^b
SCP (°C)	−12.6 $\pm$ 0.3 ^a	−9.3 $\pm$ 0.2 ^b	−5.7 $\pm$ 0.2 ^c
Heat of crystallization (°C)	$\leq 0.8^a$	$\leq 0.3^b$	$\leq 0.2^b$
Heat of crystallisation (Sec.)	77 $\pm$ 4 ^a	30 $\pm$ 3 ^b	23 $\pm$ 2 ^c
LLT ₅₀ (°C)	−14.0 $\pm$ 0.2 ^a	−10.2 $\pm$ 0.2 ^b	−6.3 $\pm$ 0.1 ^c
LLT ₉₅ (°C)	−22.1 $\pm$ 0.2 ^a	−12.6 $\pm$ 0.1 ^b	−9.8 $\pm$ 0.2 ^c

Means in the same column followed by different letters are significantly different ($P < 0.05$, ANCOVA, Tukey's HSD)

^{*}wet weight ^{**}cuticular permeability

(M) denotes surface area estimation using Meeh's formula, (H) denotes surface area estimation using Haagsma's formula

3.4.3 Body water and lipid content of the structural pests

The water balance profiles of the termite species were estimated gravimetrically. Compared with subterranean termite species, wet and dry body mass of *I. minor* workers were significantly greater ($F_{(2,51)} = 155.009$, $P < 0.0001$; $F_{(2,51)} = 2147.163$, $P < 0.0001$, respectively) and %TBW was significantly less ($F_{(3,50)} = 58.802$, $P < 0.0001$). Wet body mass ranged from 8.48 to 10.89 mg, 2.93 to 3.30 mg, and 1.83 to 2.33 mg, and dry body mass varied between 2.48 and 3.09 mg, 0.55 and 0.77 mg, and 0.39 and 0.45 mg, respectively. The mean %TBW was $72.0 \pm 0.6\%$, $78.6 \pm 0.5\%$ and $80.1 \pm 0.3\%$ for *I. minor*, *C. formosanus*, and *R. speratus*, respectively. The percentage of total body lipids for *I. minor* varied between 24.9% and 31.3% of dry mass, which was ca. 29.3% to 39.1% greater than that for *R. speratus* and *C. formosanus* ($F_{(3,50)} = 171.821$, $P < 0.0001$; Table 3.1). As a proportion of wet mass, %TBW, dry mass, and total lipids were not significantly different among the colonies for individual species ($P > 0.05$). The CP values of *I. minor* were significantly lower than those of the subterranean termites. The CP values of *I. minor* were $2.12 \pm 0.10 \mu\text{g cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$ using Meeh's formula ($F_{(3,50)} = 2325.847$, $P < .0001$) and $3.15 \pm 0.17 \mu\text{g cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$ using Haagsma's formula ($F_{(3,50)} = 409.903$, $P < 0.0001$). For the subterranean termite species, the difference in CP values was not significant between *C. formosanus* and *R. speratus* ($t_{34} = -0.682$, $P = 0.500$ for Meeh's formula, and $t_{(34)} = 0.649$, $P = 0.5212$ for Haagsma's formula) when wet body mass was considered (Table 3.1). For *I. minor*, the cumulative %TBW loss increased linearly over the 24 h desiccation; however, for both subterranean termite species, the cumulative %TBW loss increased curvilinearly with a time of desiccation (Fig. 3.2). Based on the post-hoc test, the %TBW loss rate of $0.29 \pm 0.02\% \text{h}^{-1}$ of *I. minor* workers was significantly less than the rates for *C. formosanus* ($3.83 \pm 0.02\% \text{h}^{-1}$) and *R. speratus* ($4.11 \pm 0.02\% \text{h}^{-1}$).

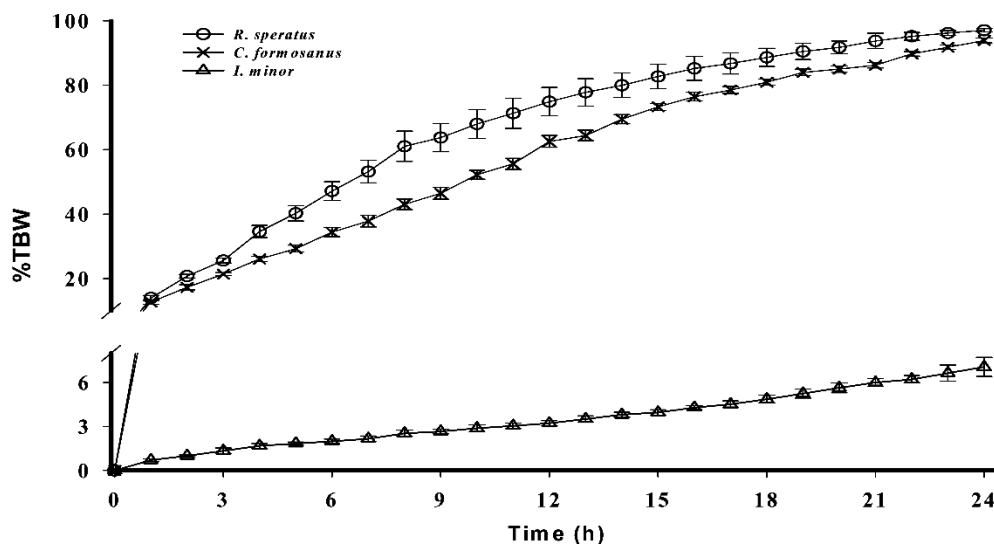


Figure 3.2 Relationship of mean cumulative %TBW lost with time for three termite species

The mean dry mass of termite workers were as follow: *I. minor* (2.78 ± 0.04 mg), *C. formosanus* (0.64 ± 0.01 mg), and *R. speratus* (0.41 ± 0.01 mg). For the dry mass of the colonies, the differences were not significant (*I. minor*: $F_{(2,3)} = 2.0133$, $P = 0.2790$; *C. formosanus*: $F_{(2,3)} = 0.4815$, $P = 0.6586$; *R. speratus*: $F_{(2,3)} = 2.3333$, $P = 0.2448$).

Incisitermes minor contained the lowest percentage level of trehalose, which was significantly different from that of the subterranean termite species ($F_{(3,14)} = 86.522$, $P < 0.0001$; Table 4.1). Trehalose, as a known cryoprotectant of dampwood termites (Lacey et al., 2010), was significantly greater in *C. formosanus* with $0.93 \pm 0.06\%$ of dry body mass. By contrast, the trehalose content in *I. minor* was $0.14 \pm 0.01\%$ of dry body mass.

3.4.5 Analysis of cuticular lipids

The mean values of cuticular lipids extracted from the three structural termite pests calculated as a percentage of the dry mass of individuals are shown in Table 3.1. The values of extracted

cuticular lipids varied between 14.5% and 20.4% of dry mass. The cuticular lipid levels of *C. formosanus* and *R. speratus* as a proportion of dry mass were significantly less than that of *I. minor* ($F_{(3,50)} = 267.309$, $P < 0.0001$). Between the subterranean termite species, the level of cuticular lipids in *C. formosanus* ($9.10 \pm 1.87\%$ of dry mass) was approximately 10% greater than that of *R. speratus* ($7.28 \pm 1.47\%$ of dry mass). The abundance of cuticular lipids in *I. minor*, based on *n*-hexane extraction, was consistent with the results of ion chromatograms.

The general pattern of the chromatograms for *C. formosanus*, *I. minor*, and *R. speratus* and the relative amounts of cuticular hydrocarbons are shown in Table 3.2. The mixture of cuticular hydrocarbons of *I. minor* included those with from 21 to 34 carbons in the parent chain. *n*-alkanes were identified for *n*-C₂₃, *n*-C₂₄, *n*-C₂₅, *n*-C₂₆, *n*-C₂₇, and *n*-C₂₉, which represented over 34.8% of the total hydrocarbons. Dimethylalkanes were the most predominant hydrocarbon at ca. 28.0% of the total hydrocarbons. The most abundant dimethylalkanes was 12,16-dimeC₃₁, which accounted for ca. 13.9% of the total hydrocarbons. Mono-methylalkanes were another abundant class of hydrocarbons, representing 18.6% of the total hydrocarbons. In *I. minor*, the early eluting compounds consisted mostly of *n*-alkanes and branched methylalkanes, whereas all dimethylalkanes were primarily in late-eluting compounds. After the C₃₄, there was ca. 15.9% hydrocarbons were identified as unsaturated hydrocarbons.

Coptotermes formosanus contained 50% relatively less hydrocarbons than *I. minor*. *n*-alkanes (C₂₅, C₂₆, and C₂₇) were rare and composed only 5.3% of the total hydrocarbons. The predominant class of hydrocarbons was 2-meC₂₅, representing ca. 15.4% of the total hydrocarbons. Monomethylalkanes were the most abundant and composed more than 56.6% of the total hydrocarbons. Three of dimethylalkanes, 9-,16 dimeC₂₆, 9-,13-demeC₂₇, and 9-,13-dimeC₂₈ were identified, representing ca. 25.5% of the total hydrocarbons. However, the percentage was difficult

to determine because of the incomplete separation of peaks, which included 2-meC₂₇. Both peaks represented ca. 14.6% of the total hydrocarbons. Unsaturated hydrocarbons were detected from *C. formosanus* (ca.11.2%).

The general patterns of the chromatograms of *R. speratus* were similar to those of *C. formosanus*. All hydrocarbons were in the early eluting compounds (C₂₃-C₂₇), and trimethylalkanes and unsaturated compounds were not identified in *R. speratus*. The most abundant class of hydrocarbons was monomethylalkanes, which composed ca. 51.5% of the total hydrocarbons. Internally branched monomethylalkanes, 2,8-, and 3,7-dimethylpentacosane, were the predominant class of hydrocarbons, representing ca. 28.1% of the total hydrocarbons. All *n*-alkanes in *R. speratus* combined were ca. 18.2% of the total hydrocarbons.

Table 3.2 Composition of cuticular hydrocarbons (mean $\pm$ SD) of workers of three termite species.

Hydrocarbon	ECL ^a	Relative amount (%)		
		<i>I. minor</i>	<i>C. formosanus</i>	<i>R. speratus</i>
<i>n</i> -C22	2200	-	-	0.5 (0.2)
<i>n</i> -C23	2300	1.1 (0.2)	-	1.5 (0.6)
2-meC23	2364	4.4 (1.1)	2.7 (1.8)	2.3 (1.1)
3-meC23	2374	2.1 (0.9)	-	4.2 (1.3)
<i>n</i> -C24	2400	3.8 (1.5)	-	3.9 (0.8)
2-meC24	2463	1.6 (1.2)	-	10.9 (2.3)
<i>n</i> -C25	2500	12.1 (4.2)	1.4 (0.8)	8.2 (2.1)
9, 11-meC25	2535	-	-	10.1 (2.7)
2-meC25	2564	3.6 (2.1)	15.4 (3.5)	6.8 (1.0)
3-meC25	2574	1.5 (0.7)	9.2 (1.9)	5.6 (1.9)
2-, 8- dime and/or 3-, 7-dimeC25	2591	-	-	28.1 (6.3)
<i>n</i> -C26	2600	3.4 (0.6)	1.5 (0.4)	-
11-, 13-meC26	2635	-	2.2 (0.8)	-
2-meC26	2664	3.1 (1.3)	3.7 (1.9)	-
3-meC26	2674	-	1.8 (0.5)	
9- and 16-dimeC26	2683	-	3.6 (1.1)	2.9 (0.6)
<i>n</i> -C27	2700	12.9 (2.2)	2.8 (1.4)	4.1 (1.3)
7-,9-, 11-,13-meC27	2735	-	15.1 (4.9)	2.7 (1.6)
9-,13-dimeC27	2768	-	12.3 (3.5)	7.8 (2.6)

Table 3.3 Composition of cuticular hydrocarbons (mean $\pm$ SD) of workers of three termite species- Continued

Hydrocarbon	ECL ^a	Relative amount (%)		
		<i>I. minor</i>	<i>C. formosanus</i>	<i>R. speratus</i>
3-meC27	2774	-	3.7 (1.9)	0.4 (0.2)
<i>n</i> -C28	2800	-	0.4 (0.2)	-
9-, 13-dimeC28	2868	-	4.7 (1.5)	-
<i>n</i> -C29	2900	1.5 (0.8)	0.6 (0.2)	-
9-,11-,13-,15-meC29	2938	-	2.8 (0.7)	-
9-,13-dimeC29	2966	2.4 (1.4)	4.9 (1.2)	-
10-,14-dimeC30	3067	1.6 (0.8)	-	-
11-meC31	3133	2.2 (0.7)	-	-
12,16-dimeC31	3170	13.9 (2.5)	-	-
9-,11-meC32	3233	0.4 (0.2)	-	-
12-,16-dimeC32	3262	2.9 (1.5)	-	-
9-,11-meC33	3334	2.3 (0.9)	-	-
3-,13-dime and/or 12-,16-dimeC33	3368	6.1 (2.2)	-	-
12-,16-dimeC34	3468	1.2 (0.3)	-	-
Unsaturated	> 3458	15.9 (2.6)	11.2 (2.3)	-

^a ECL; Equivalent chain length

C. formosanus consisted relatively 50% fewer hydrocarbons than *I. minor*. *n*-alkane (C₂₅ C₂₆ and C₂₇) present were rare and consisted of only 3.6% of the total hydrocarbons. The predominant class of hydrocarbons was 9-,11-,13-meC₂₇, representing ca. 40.3% of the total hydrocarbons. Monomethylalkanes were the most abundant, comprising more than 81.8% of the total hydrocarbons. 9,13-dimethylheptacosane was identified as single dimethylealkanes. However, it was hard to account the percentage since the incomplete separation of peaks which are included 2-meC₂₇. Both peaks representing ca.14.6% of the total hydrocarbons.

We identified the general patterns of the chromatograms of *R. speratus* were similar to that of *C. formosanus*. All hydrocarbons comprised in the early eluting compounds (C₂₃-C₂₇) and no trimethylalkanes and unsaturated compounds were identified in *R. speratus*. The most abundant class of hydrocarbons was monomethylalkanes; comprising ca. 51.5% of the total hydrocarbons. Internally branched monomethylalkanes, 2,8-, and 3,7-dimethylpentacosane, was the predominant class of hydrocarbons, representing ca. 26.3% of the total hydrocarbons. All of the *n*-alkanes in *R. speratus* combined represented ca. 20.3% of the total hydrocarbons.

3.5 Discussion

A degree of cross-tolerance to desiccation and low temperature is demonstrated in many studies (Bayley et al., 2001; Danks, 2000; Gibbs, 1999; Sinclair et al., 2007). As noted above, workers of *I. minor* had a cuticular permeability that was significantly less than that of the subterranean termites, at least by 13-fold. Similar to the increased tolerance to desiccation, based on thermal tolerance, the workers of *I. minor* were also more resistant to cold temperatures. In contrast to drywood termites, subterranean termites can escape unfavorable conditions when necessary; therefore, it was reasonable to assume that the desiccation and cold hardness tolerance of these subterranean species would be lower than that of the drywood termite. Termites can overcome

these environmental stress with biological variables that include balancing body water content, generating polyols and carbohydrates, and altering lipid composition (Choi and Yoshimura, 2016a; Hu et al., 2012; Lacey et al., 2010; Mitchell et al., 1993).

Balancing body water content is improved survivability in cold temperature since body tissues are damaged by a growing ice lattice. Therefore, to prevent damage to tissues and organs, insects either supercool their body water and avoid freezing or concentrate solute in their body fluids by dehydration (Bale, 2002; Denlinger and Lee Jr, 2010; Salt, 1961; Sinclair et al., 2003a). As described in many other studies, a linear relationship between body water content and thermal responses was also observed in our study. Comparing the thermal responses of the three termite species (Table 3.1), *I. minor* was more resistant to cold temperature and generated significantly more heat for release longer than subterranean termite species to remain active (Fig. 3.3).

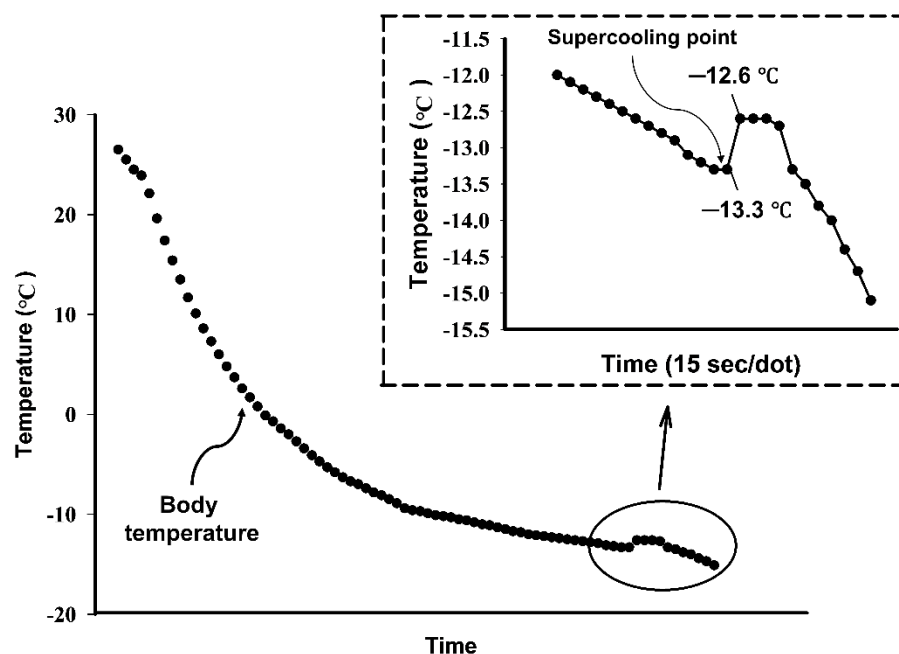


Figure 3.3 Diagram representing the SCP and latent heat fusion of the western drywood termite, *I. minor*

Supercooling is the most common mechanism used by insects to avoid freezing (Block et al., 1990), with the capacity for supercooling inversely related to water volume (Angell et al., 1982) and metabolites in the hemolymph of an insect (Denlinger and Lee Jr, 2010). Consequently, individuals with lower body water content may be able to depress SCPs and LLTs much less than those of subterranean termite species. We observed that the mean SCPs of all three species were slightly below the lethal low temperature for 50% mortality, which indicated that all three structural termite pests in Japan are most likely freeze-avoiding insects.

Several different carbohydrates and polyols are found in sufficiently high concentrations to enhance cryoprotection properties in insects. As noted by Lacey et al. (2010) and Choi et al. (2016), a high level of trehalose acts as a cryoprotectant in *Porotermes adamsoni*, *Stolotermes victoriensis*, and *Reticulitermes speratus*. Trehalose is a non-reducing disaccharide found in high concentrations as the primary hemolymph sugar in insects (Becker et al., 1996). Trehalose interacts with phospholipids through hydrogen bonding, which increases the capability to survive osmotic stress (Crown et al., 1984) and also protects cellular membranes from denaturation caused by cold temperatures and desiccation (Elbein et al., 2003). However, based on the analysis of trehalose in this study, trehalose did not function as a natural cryoprotectant in *I. minor*. The maximum trehalose concentration in *C. formosanus* (0.93% of dry mass) coincided with the SCP ($-9.3 \pm 0.2^{\circ}\text{C}$) and the peak of LLT₉₅ ($-12.6 \pm 0.1^{\circ}\text{C}$). By contrast, trehalose accumulated little more than half this level (0.41%) in *I. minor*; however, comparatively, the SCP was approximately 74.0% less, and the peak of LLT₉₅ was approximately 56.8% less (Table 3.1). Consequently, no linear correlation was observed between trehalose content and cold tolerance in *I. minor*. The low concentration of trehalose in *I. minor* might be the result of an inherited aspect of the physiological ecology of drywood termites. Commonly, in freeze-avoiding insects, the concentrations of

carbohydrates and polyols usually increase beginning in autumn (Bale, 2002). Additionally, insects in cold habitats typically contain a high level of trehalose (Fields et al., 1998). Hence, *I. minor*, which is native to the desert and semi-arid southwestern U S and adapted to living entirely with air-dried timbers in hot and dry areas of buildings, might not have developed a competitive trehalose adaptation for a cold environment. Instead, *I. minor* is highly evolved in the regulation of water balance and properties of cuticular resistance (i.e., more cuticular lipids and long-chain hydrocarbons in late-eluting compounds) since external sources initiate freezing by inoculation through the cuticle.

Many researchers recognize the importance of lipids and their roles in physiological processes. Thus, many studies report that lipid amounts are influenced by several factors, including environmental temperature, nutritional state, and stage of development (Beenakkers et al., 1985; Craig et al., 1995; Lee Jr, 1991; Overgaard et al., 2007). Among other factors, several authors note that changes in lipid stores are associated with seasonal changes in termites (Choi et al., 2016; Lacey et al., 2010), bark beetles (Lombardero et al., 2000), cockroaches (Appel et al., 1983) and other insects (Lee Jr, 1991). The lipid phase of membranes effectively prevents the propagation of extracellular ice into the cells and organs (Zachariassen, 1985) by modifying the proportion of unsaturated fatty acids (Lee et al., 2006); consequently, lipids function as a natural cryoprotectant in insects. Based on our analysis of total lipid content, a high content of cuticular lipids provided better protection against cold temperatures. Within subterranean termite species, the thermal response of *C. formosanus* was greater than that of *R. speratus* even with a less total body lipid content. Total body lipid concentration in *R. speratus* ($27.32 \pm 0.65\%$ of dry mass) coincided with the SCP ($-5.7 \pm 0.2^{\circ}\text{C}$) and the peak of LLT₉₅ ($-9.8 \pm 0.2^{\circ}\text{C}$). In comparison, although *C. formosanus* had a total body lipid content that was ca. 4.5% less (26.04% of dry mass), the SCP was ca. 61.1%

less, and the peak of LLT₉₅ was ca. 78.2% less (Table 3.1). Based on our results, the correlation between cuticular lipid concentration and thermal responses was apparently adequate to explain the greater freeze tolerance in *C. formosanus*, and the approximately 17.9% greater concentration of cuticular lipids in *C. formosanus* significantly influenced their thermal response against cold temperature. The role of cuticular lipids as a cryoprotectant has been documented (Sømme, 1982), and in a report, the cuticular lipids of insects inhibit inoculative freezing because inoculation freezing is initiated by external ice across the cuticle. Furthermore, extremely low cuticular permeability may restrain inoculative freezing (Lundheim and Zachariassen, 1993). Based on the results of our hydrocarbon analysis, unsaturated and long-chain hydrocarbons (> 27 Carbons) tended to influence cold tolerance positively. Unfortunately, this statement cannot be supported by direct references that examined the correlation between chain length and cold tolerance. However, in most studies of insects collected in xeric environments and of individuals stressed by xeric conditions, increased proportions of long-chain compounds are found in the cuticular hydrocarbons (Collins et al., 1997; Howard et al., 1995; Woodrow et al., 2000). Consequently, long-chain compounds may influence the cold tolerance of *I. minor* because of the degree of cross-tolerance between desiccating and cold conditions.

To our knowledge, this study is the first to compare and identify a correlation between the natural cryoprotectants and cold tolerance of western drywood termites. Metabolite and thermal response data indicated that the low body water content and hardened cuticle structure (i.e., a greater concentration of cuticular lipids with more long-chain compounds) of *I. minor* might have resulted in the lower SCPs and LLTs compared with those of the subterranean termite species.

CHAPTER 4: Physiological adaptations to environmental stresses of termites (Isoptera) species of Japan

Among the environmental stress (low temperature and desiccation) in nature that termites encounter, they evolve a range of biological adaptations that work in concert to improve their chances of survival in both environmental stresses. Many studies find that the tolerance of termites for unfavorable conditions is usually linked to physiological adaptations that likely evolved to regulate body water content and synthesize cryoprotectant metabolites to stabilize insect membranes during hypertonic environment exposure. The first part of this chapter presents evidence that the physiological adaptation that protects against cold stress also protects against desiccation, and that the responses to cold and dehydration likely evolved as cross-tolerance.

From the results from chapter 3 and 4a, a natural cryoprotectant, trehalose, did not show the significant impact on the cold and desiccation tolerance of western drywood termite. It may be related to the natural habitat of termite; therefore, in the second part of this chapter, the carbohydrates, the total lipids, and the body water content between summer- and winter-acclimatized moisture termites, *Reticulitermes speratus* (Kolbe), in three latitudinal regions were compared and determined whether northern populations possess an additional physiological capacity to acclimate to cold temperatures. Since cold-tolerant insects can also acclimate to cold temperatures by altering carbohydrates (trehalose and glucose) and their lipid content to increase their survivability during slower and less abrupt seasonal changes in temperature.

A) Determination of physiological adaptation to cope with desiccation stress and efficacy of sublimation of CO_{2(s)}, a paper has been published as below:

Choi, B., and T. Yoshimura (2016) Effects of Induced High Carbon Dioxide and Desiccated Atmospheres on the Water Loss and Survival of Subterranean and Invading Drywood Termites. *Journal of Economic Entomology*. 109 (2): 753-761.

B) Determination of physiological adaptation to cope with low temperature stress in three contrast latitudes, a paper has been published as below:

Choi, B., and T. Yoshimura. (2016). Do northern populations of *Reticulitermes speratus* (Kolbe) possess an additional physiological capacity to cold acclimate that enhances cold tolerance during the winter? *Journal of Asia-Pacific Entomology* 19: 643-649

Publication A Effects of Induced High Carbon Dioxide and Desiccated Atmospheres on the Water Loss and Survival of Subterranean and Invading Drywood Termites

4.1 summary

Termites have evolved a variety of morphological, physiological, and behavioral adaptations that together increase the chances of survival in hostile environments. Morphological advantages (i.e., a relatively large body size, a large mass of reserves that are metabolized by reducing body water mass, a mass of cuticular lipids, and cuticular hydrocarbons) increase the cuticular resistance to water loss under desiccating conditions. However, termites are incapacitated when exposed to high levels of CO₂ and can be eradicated at sufficiently high levels. Based on the results of this study, in an artificially induced high CO₂ atmosphere, the loss of body water from drywood termites increased, which led to increased rates of mortality. Although the mechanism for the increased loss of body water under high CO₂ conditions was not determined, for practical application, the time required for fumigation can be reduced because of the increase in the rate of body water loss from drywood termites in high CO₂ conditions.

4.2 Introduction

From the time the borders of Japan opened to the world in 1868, 433 species of insects have been introduced to the country (Mito and Uesugi, 2004). These introductions include three species of drywood termites (i.e., *Cryptotermes brevis*, *Incisitermes immigrans* and *Incisitermes minor*), which represent slightly less than 0.7% of the total number of invaded insect species according to the Japanese Ministry of the Environment (JME, 2014). Among these drywood termite invaders, *I. minor* most commonly infests structures in Japan and causes damages worth \$8 million the US or 1% of the total termite economic impact (Verma et al., 2009).

Because of the ecology, adaptive biology, and cryptic lifestyle, in addition to increased transport with intercontinental travel and commerce, the potential is high for drywood termites to spread and proliferate in other parts of Japan. For example, the discovery of an *I. minor* infestation in Japan was first reported in 1976 (Mori, 1976), with the termite likely introduced in the moving shipment of a Japanese family moving from Hawaii to Tokyo. After that, *I. minor* appeared sporadically throughout the country, with apparent relocation by human activity. In the most recent infestation report from the Japan Termite Control Association, 24 out of 47 prefectures were infested with *I. minor*, and the termite was considered a structure pest in the Kansai regions (JWPA, 2013). Because the termite has been in Japan for only the relatively short period of fewer than 40 years, the economic impact of this drywood termite remains low. However, a half century passed before *I. minor* became an economically important structure pest in the United States, and therefore, without proper integrated pest management (IPM), this pest may become a serious problem within the next 10-20 years in Japan (Hunt, 1959; Scheffrahn and Su, 2014).

Numerous comparative studies of adaptations show that termites have evolved a diversity of morphological, physiological, and behavioral adaptations that together increase the chances for termite survival in hostile environments. In several studies, termites effectively manage evaporative water loss with behavioral (e.g., burrowing activity and aggregation), morphological (e.g., cuticle thickness, amount of cuticular lipids, cuticular hydrocarbons, and presence of cement layer, among others), and physiological adaptations (e.g., control of water content in the labial gland, content of stored energy, and control of rates of water loss and metabolism) (Abe et al., 2000; Cabrera and Kamble, 2001; Collins, 1969; Gautam and Henderson, 2014; Haverty et al., 1996; Shelton and Appel, 2001; Sponsler and Appel, 1990; Woodrow et al., 2000). These biological adaptations increase the resistance of individual termites to desiccation and are

described by the general character of cuticular permeability (CP). High CP indicates that insects are adapted to mesic habitats; whereas low CP values indicate an adaptation to xeric habitats. In these xeric habitats, low CP reduces the physical damage and mechanical distortion caused by water loss and provides sufficient time for biochemical adaptations (Rosengaus et al., 2003; Rust et al., 1979; Sponsler and Appel, 1990). The CP values were determined for two economically important termite species in Japan, *Coptotermes formosanus* Shiraki and *Incisitermes minor* (Hagen): *I. minor* workers, CP = 1.5–2.3 $\mu\text{g H}_2\text{O cm}^{-2} \text{ mm Hg}^{-1}\text{h}^{-1}$; and *C. formosanus* workers, CP = 19.33–25.61 $\mu\text{g H}_2\text{O cm}^{-2} \text{ mm Hg}^{-1}\text{h}^{-1}$ (Rosengaus et al., 2003; Rust et al., 1979; Sponsler and Appel, 1990).

The principal control measure against drywood termite infestation in North America is fumigation with sulfuryl fluoride (Delate et al., 1995). However, sulfuryl fluoride was recently shown to have effects on the atmosphere, which renewed interest in the inert fumigants carbon dioxide and nitrogen (Lewis and Forschler, 2014). Based on previous investigations, both inert fumigants are effective for the control of drywood termites (Delate et al., 1995; Paton and Creffield, 1987; Rust et al., 1996). However, for large-volume operations, carbon dioxide is the gas of choice because of much lower costs and less stringent sealing requirements (Selwitz and Maekawa, 1998). Although the effectiveness of CO₂ has been confirmed in a number of studies, CO₂ gas has not been applied to a drywood termite infestation because of the long treatment times.

In this study, we investigated the immediate effects of an atmosphere with experimentally induced high levels of carbon dioxide on the dehydration of termites because accelerated desiccation and increases in mortality rate are positively correlated with body water loss. First, we examined several aspects of the water balance, including the CP, percentage of total body water (%TBW) and cumulative %TBW loss, and of total lipids, including the quantities of body and

cuticular lipids and the composition and properties of cuticular hydrocarbons, of *I. minor* and two economically important subterranean termite species for comparison. Second, to determine whether these biological adaptations influenced the cuticular resistance, we used correlations between the adaptations and the lethal total body water loss for 50% and 95% mortality (LTL₅₀ and LTL₉₅). The termites were examined in two controlled atmospheres, one desiccated and one with high CO₂.

4.3 Materials and Methods

4.3.1 Insects

Three colonies of each termite species were collected for this study. The colonies of *Reticulitermes speratus* were collected from Japanese black pine, *Pinus thunbergii*, on the Kyoto University-Uji campus, Japan. The colonies of *C. formosanus* were from three laboratory colonies maintained at the Deterioration Organisms Laboratory (DOL) of the Research Institute for Sustainable Humanosphere (RISH), Kyoto University. The colonies of *I. minor* were collected from infested structural timbers of residential houses in Wakayama Prefecture, Japan. The individuals of each termite species were carefully removed from infested logs or branches for biological analysis and determination of the lethal total body water loss to induce 50% and 95% mortality (LTL₅₀ and LTL₉₅) in experimentally induced low relative humidity and high CO₂ atmospheres.

4.3.2 Analysis of water balance

A total 18 replications (a mixture of large larvae, psudergates, and nymphs), (two replicates/colony/species) were used, and each replication contained 15 termites. Total body water (%TBW), cumulative %TBW loss, and cuticular permeability (CP) were determined gravimetrically under desiccating conditions as described by Appel and Tanley (1999), with slight

modifications. The CP values were determined during the first 2 h of exposure, which is the period of maximal water loss. Before calculating CP, the surface area for each termite species was calculated with the following two formulas:

Meeh's formula (1879): $S = 12M^{2/3}$, where S = surface area (cm²) and M = initial weight (g);

Haagsma's formula (1996): $y = 0.0886 + (26.85*x) - 214.21*x^2$, where y = surface area (cm²) and x = initial weight (g).

Cuticular permeability (CP) was defined as water loss [initial weight – weight after first 2 h of the experiment] (µg H₂O) per unit surface area (cm²) per unit time (h⁻¹) per unit saturation deficit (mm Hg⁻¹). For the saturation deficit, 26.95 mm Hg was used because the tests occurred at only one temperature (28 °C) and RH (< 2%).

4.3.3 Estimation of total lipids (cuticular lipids and body lipids)

With two replicates/colony/species, 18 total replications were used with termites from the identical colonies used for the water balance analysis. Live weights were measured to the nearest 0.01 mg using a semi-microanalytical balance (A&D-GH252, Tokyo, Japan) for the estimation of cuticular lipids. To obtain dry weights, termites were placed in an oven at 60 °C for 48 h. The oven-dried termites were used to extract cuticular lipids following the procedure described by Parkash et al. (2008), with a modification of the dipping time. Pre-weighed termites were placed in a glass vial (5 ml), 2 ml of HPLC-grade hexane was added, and the cuticular lipids were extracted for 10 min. After extraction, the exoskeletons in the extraction solution were removed by filtration through an Omnipore membrane filter (0.2 µm JG type; Darmstadt, Germany). The extract was dried at 60 °C for 48 h to obtain the dry weight of the exoskeletons without the cuticular

lipids. The percentage of cuticular lipid was calculated as $([\text{Initial dry weight} - \text{dry weight after extraction}]/\text{Initial dry weight}) \times 100$.

The cuticular lipid mass per termite was estimated in mg per unit surface area (for the surface area calculated with the formulas of both Meeh and Haagasma) as follows:

Cuticular lipid mass per unit surface area (mg cm^{-2}) =

$\text{Initial dry weight} - \text{dry weight after extraction} / \text{initial dry weight} * \text{surface area}$

The cuticular lipid mass extracted from the termites was used to estimate the total body lipid content. The procedure described by Foo et al. (2011) was used, with slight modification. Dried termites were pre-weighed and separated into three pieces, which were placed in a glass vial (5 ml) with 2 ml of chloroform added. The vials were left standing for 48 h for total body lipid extraction. As described for the cuticular lipid measurement, exoskeletons in the extraction solution were removed by filtration through an Omnipore membrane filter ($0.2 \mu\text{m}$ JG type; Darmstadt, Germany). To determine the total lipid content, the extract was dried at 60°C for 48 h to obtain the exoskeleton dry weight without the total lipids. Percentage total lipids were calculated as $([\text{dry weight before extraction} - \text{dry weight after extraction}]/\text{dry weight before extraction}) \times 100$.

4.3.4 Analysis of cuticular hydrocarbons

The cuticular lipids of individual termites (45 workers for *I. minor*, 270 for *C. formosanus*, and 300 for *R. speratus*) were extracted by immersion in 10 ml of *n*-hexane (Nacalai Tesque, suitable for HPLC analysis) for 10 min. After extraction from the termites, the hydrocarbons were separated from other components by pipetting the extract through 4 cm of activated BioSil-A (silica gen, Nacalai tesque, 230-400 mesh) in Pasteur pipet minicolumns (5 mm). The resulting extracts were evaporated to dryness under nitrogen and were re-dissolved in 10 μl of *n*-hexane for

gas chromatography-mass spectrometry (GC-MS) analyses. The analysis of cuticular hydrocarbons was performed on a GC-MS (QR5000; Shimadzu, Kyoto, Japan) using a wide-bore capillary column (30 m x 0.25 mm ID, HP-1) as described in Haverty et al. (1988). The carbon number and the retention times compared with external n-alkane standards were used to identify the unknown hydrocarbons (Restek, Bellefonte, PA). The total hydrocarbons were then automatically quantified by summing the area and proportion of each peak.

4.3.5 Desiccation atmosphere

Immediately after collection from infested wood, 18 total replications of termites (as per the water balance analysis) were weighed and placed in uncovered 15 ml glass vials inside an airtight chamber (a 25 x 20 x 20 cm polyethylene container) that contained 1.0 kg of silica gel (pre-dried at 110 °C for 24 h) at 0–2% relative humidity (RH). The temperature and RH were monitored with a thermohygrometer (T&D TR-72U; Tokyo, Japan). The desiccation chamber was placed in an incubator (EYERA KCL-2000, Tokyo, Japan) at 28 °C. Total body water loss and mortality were recorded hourly for 24 h. Mortality was defined as the failure to show any perceptible movement. After 24 h, the termites were removed from the desiccation chamber and dried at 60 °C for 48 h to obtain total body water content.

4.3.6 Induced high carbon dioxide atmosphere

The identical colonies were used to collect and prepare replicates to determine cumulative %TBW loss and mortality. To determine cumulative %TBW loss, the method was identical to that used in the desiccation evaluation without any silica gel (six replicates/species for a total of 18 replications). Because of the long recovery time from CO₂ narcosis, a separate test was conducted to determine mortality after 10 different time intervals (1, 2, 4, 6, 8, 10, 12, 18, 21 and 24 h) with three replications for each time interval using 10 vials (15 termites/vial) for each species. This

implies 150 termites for each species were examined for each interval. During the recovery from CO₂ narcosis, the activity of the termites was observed and recorded using a video camera (HDR-XR500V; SONY, Tokyo, Japan). The recovery time was recorded at the first full locomotion of any termite in each replicate, which was defined as the movement of all six legs.

To maintain high carbon dioxide conditions (> 95%) for 24 h, gas was supplied by the sublimation of solid carbon dioxide (hereafter dry ice) (Nakagawa Ltd., Tokyo, Japan) by placing 2.5 kg of dry ice (purity 99.5%) in an airtight polyethylene cylindrical chamber (19.4 x 14.9 cm). The gas was delivered through a PVC tube (90-cm long with a 0.5-cm diameter) into the airtight chamber without silica gel. To measure the instantaneous CO₂ concentration, temperature and RH inside the chamber, a CO₂ monitor (XP-3140; New Cosmos Electric, Osaka, Japan) and a thermo-hygrometer (Model TR-72U; Tokyo, Japan) were used.

4.3.7 Statistical analyses

Before analysis of variance (ANOVA), all percentage data were arcsine square root transformed. To evaluate differences between termite species, ANOVA was performed, and means were separated using Tukey's honestly significant difference (HSD) tests. The differences in the desiccated and induced high CO₂ atmospheres were explored using the dependent Student *t*-test. To determine the lethal total body water loss that induced 50% and 95% mortality of the termite species, probit analyses were conducted to correlate the change in mortality and exposure time. All statistical analyses were conducted using JMP 11.0.0, with significance at the 95% confidence level (SAS Institute, 2013).

4.4 Results

4.4.1 Analysis of water balance

The fresh and dry body mass of the drywood termite *I. minor* were significantly greater ($F = 372.000$, $df = 17$, $P < .0001$; and $F = 569.0989$, $df = 17$, $P < .0001$, respectively) and the %TBW was significantly lower than that of the subterranean termite species ($F = 62.023$; $df = 17$; $P < .0001$). Fresh body mass ranged from 2.39 to 2.71 mg, 3.73 to 4.49 mg, and 9.79 to 12.48 mg; dry body mass ranged from 0.45 to 0.59 mg, 0.86 to 1.01 mg, and 2.93 to 3.57 mg; and %TBW ranged from 77.1% to 82.5%, 77.5% to 79.4%, and 68.3% to 72.1% for *R. speratus*, *C. formosanus* and *I. minor*, respectively. Within a species, significant differences were not detected for fresh body mass, dry body mass or %TBW among the three colonies ($P > 0.05$). The CP values among termite species were significantly different using either Meeh's ($F = 267.110$; $df = 17$; $P < .0001$) or Haagsma's ($F = 242.379$; $df = 17$; $P < .0001$) formulas; however, no differences were detected among the colonies for individual species ($P > 0.05$; Table 4a.1).

4.4.2 Estimation of total lipids (cuticular lipids and body lipids)

Overall, *I. minor* had a larger total lipid mass than that of the subterranean termites ($F = 234.307$; $df = 17$; $P < 0.0001$). The percentage total lipids (cuticular lipid and remaining body lipid) for *I. minor* ranged from 12.3% to 14.4% (fresh weight). Of the total lipids in *I. minor*, approximately 61.5% were body lipids with the remaining portion cuticular lipids (Table 4a.1). The percentage total lipids of *C. formosanus* and *R. speratus* ranged from 4.4% to 5.7% and 4.7% to 6.4% (fresh weight), respectively, and the difference was not significant between the species ($P > 0.05$). Both species of subterranean termites had higher proportions of body lipids and lower proportions of cuticular lipids than those of the drywood termite, *I. minor*. In a comparison of the subterranean termites, the amount of body lipids in *R. speratus* was significantly greater than that in *C.*

formosanus (Meeh: $t = 17.349$, $df = 10$, $P < 0.0001$; and Haagsma: $t = 12.033$, $df = 10$, $P < 0.0001$), whereas no significant differences were detected for cuticular lipids between the species ($P = 0.1646$).

Table 4a.1 Mean fresh and dry mass, water relations, CP, and total lipid composition (mean $\pm$ SE) of three termite species.

Parameters	<i>I. minor</i> (n = 90)	<i>C. formosanus</i> (n = 90)	<i>R. speratus</i> (n = 90)
Fresh body mass (mg)	11.19 $\pm$ 0.38 ^a	4.30 $\pm$ 0.16 ^b	2.52 $\pm$ 0.05 ^c
Dry body mass (mg)	3.33 $\pm$ 0.10 ^a	0.93 $\pm$ 0.03 ^b	0.50 $\pm$ 0.02 ^c
TBW content (%)	70.2 $\pm$ 0.01 ^b	78.3 $\pm$ 0.00 ^a	80.1 $\pm$ 0.01 ^a
CP* — Low RH	(M) 2.54 $\pm$ 0.28 ^b	(M) 29.02 $\pm$ 1.23 ^a	(M) 32.38 $\pm$ 1.19 ^a
($\mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$)	(H) 4.21 $\pm$ 0.46 ^b	(H) 43.09 $\pm$ 1.77 ^a	(H) 43.46 $\pm$ 1.71 ^a
CP — High CO ₂	(M) 6.43 $\pm$ 0.40 ^b	(M) 4.60 $\pm$ 0.42 ^a	(M) 5.33 $\pm$ 0.21 ^{ab}
($\mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$)	(H) 10.67 $\pm$ 0.67 ^b	(H) 6.97 $\pm$ 0.62 ^a	(H) 7.10 $\pm$ 0.28 ^a
Body lipids (% of fresh weight)	8.0 $\pm$ 0.04 ^a	3.0 $\pm$ 0.1 ^c	4.0 $\pm$ 0.2 ^b
Cuticular lipids (% of fresh weight)	5.0 $\pm$ 0.1 ^a	1.8 $\pm$ 0.1 ^b	1.6 $\pm$ 0.1 ^b
Cuticular lipid mass per termite (mg cm ⁻²)	(M) 0.404 $\pm$ 0.007 ^a (H) 0.244 $\pm$ 0.004 ^a	(M) 0.205 $\pm$ 0.002 ^b (H) 0.136 $\pm$ 0.001 ^b	(M) 0.154 $\pm$ 0.002 ^c (H) 0.115 $\pm$ 0.001 ^c

Means in the identical row followed by different letters are significantly different ($P < 0.05$, ANOVA followed by Tukey's HSD).

* CP = Cuticular permeability.

(M) denotes the surface area estimation using Meeh's formula.

(H) denotes the surface area estimation using Haagsma's formula.

The cuticular lipid mass per *I. minor* termite ranged from 0.382 to 0.431 mg cm⁻² (Meeh's formula) and from 0.230 to 0.260 mg cm⁻² (Haagsma's formula), which were values that were approximately 2- to 2.6-fold higher than those in the subterranean termites. In a comparison of cuticular lipid mass per unit surface area of the subterranean termites, notably, *R. speratus* had a significantly lower mass of cuticular lipids than that of *C. formosanus* (Table 4a.1). Within a

species, no significant differences in total lipids (cuticular and body lipids) were detected among the three colonies ($P > 0.05$)

4.4.3 Analysis of cuticular hydrocarbons

The gas chromatographs with the components and relative amounts of the cuticular hydrocarbons of the three termite species are shown in Fig. 4a.1 and Table 4a.2-3. From the 3 colonies of each termite species, *R. speratus* contained 2-,8-dime and/or 3-,7-dimeC₂₅, which accounted for ca. 29.7% of the total hydrocarbons and were the predominant hydrocarbons in the mixture. The *n*-alkanes (*n*-C₂₃, *n*-C₂₄, *n*-C₂₅, and *n*-C₂₇) were the other abundant compounds with ca. 17.7% of the total hydrocarbons. By contrast, in *C. formosanus*, the late eluting compounds (C₂₅-C₂₉) were the most abundant hydrocarbons, and methyl-branched alkanes and alkenes with C₂₇ or C₂₉ accounted for ca. 78.5% of the total hydrocarbons with 7-, 9-, 11-, and 13-meC₂₇ the predominant hydrocarbons in the mixture. By contrast, alkanes accounted for only 7.7% of the total hydrocarbons in the mixture.

From the 3 colonies of *I. minor*, a total of 19 components of cuticular hydrocarbons from C₂₃ to C₃₄ were identified, with an additional 10 unknown components from C₃₅ to C₄₃ detected. The *n*-alkanes (*n*-C₂₃, *n*-C₂₄, *n*-C₂₅, *n*-C₂₆, *n*-C₂₇, and *n*-C₂₉) were the most abundant compounds, with ca. 23.3% of the total. The three predominant compounds in *I. minor* were *n*-C₂₅, *n*-C₂₇, and 12,16-dimeC₃₁.

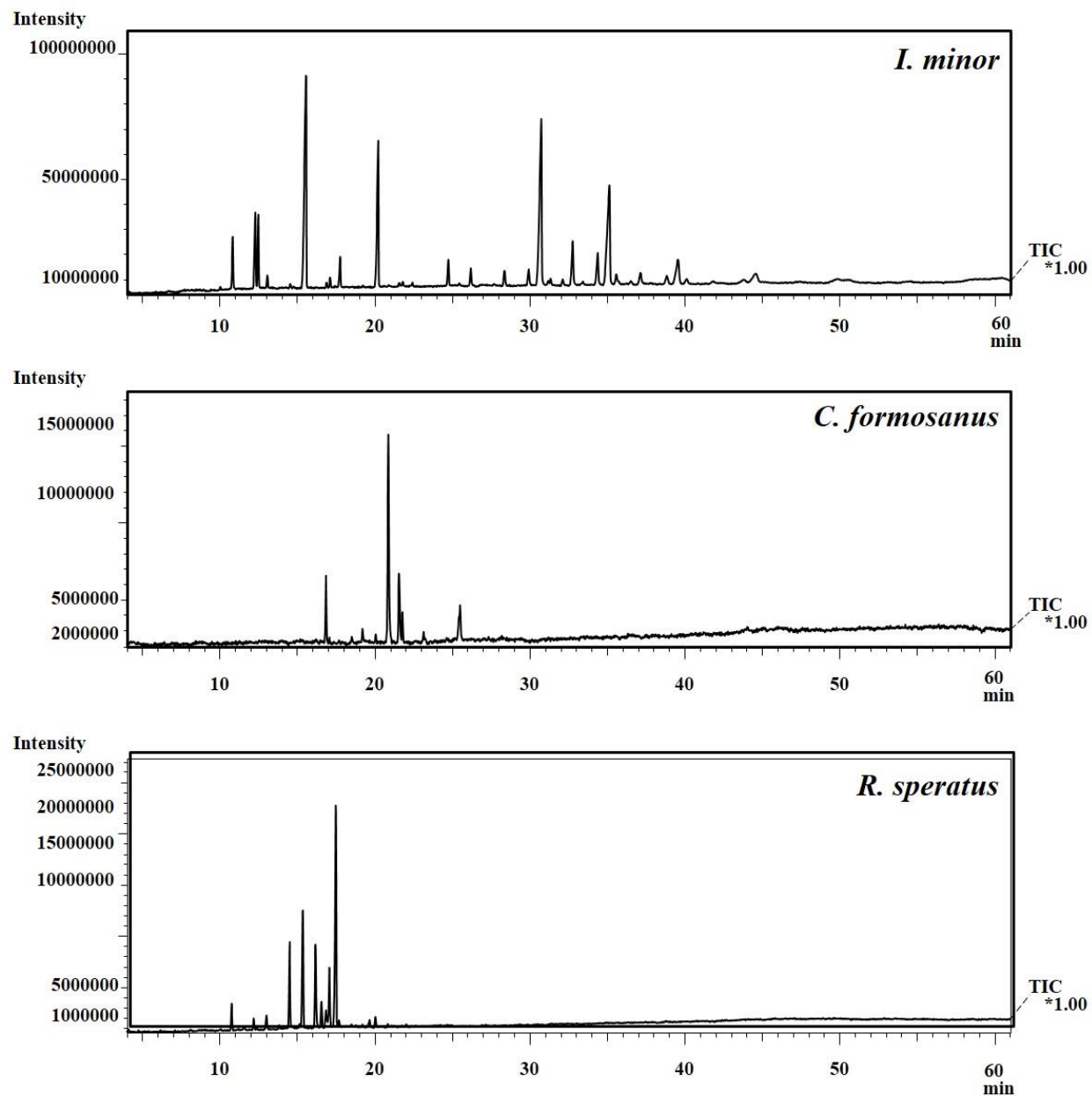


Figure 4a.1 Total ion chromatogram of the cuticular hydrocarbons of three termite species from Japan

Table 4a.2 The composition of cuticular hydrocarbons (mean $\pm$ SD) of workers of three termite species

Hydrocarbon	ECL ^a	Relative amount (%)		
		<i>I. minor</i>	<i>C. formosanus</i>	<i>R. speratus</i>
<i>n</i> -C ₂₂	2200	-	-	0.4 (0.2)
<i>n</i> -C ₂₃	2300	0.3 (0.1)	-	2.8 (0.8)
2-meC ₂₃	2364	4.2 (1.3)	2.3 (0.8)	3.3 (0.6)
3-meC ₂₃	2374	3.1 (0.9)	-	3.2 (0.3)
<i>n</i> -C ₂₄	2400	4.7 (1.3)	-	3.9 (1.0)
2-meC ₂₄	2463	2.6 (1.4)	-	7.9 (1.5)
<i>n</i> -C ₂₅	2500	12.2 (4.1)	2.1 (1.3)	7.5 (1.8)
9, 11-meC ₂₅	2535	-	-	8.8 (1.2)
2-meC ₂₅	2564	3.3 (1.8)	13.4 (2.5)	6.0 (1.4)
3-meC ₂₅	2574	1.7 (0.6)	10.0 (1.9)	7.4 (2.9)
2-, 8- dime and/or 3-, 7-dimeC ₂₅	2591	-	-	29.7 (3.7)
<i>n</i> -C ₂₆	2600	3.2 (0.8)	1.5 (0.2)	-
11-, 13-meC ₂₆	2635	-	1.8 (0.3)	-
2-meC ₂₆	2664	2.7 (1.1)	3.2 (1.9)	-
3-meC ₂₆	2674	-	2.3 (0.5)	
9- and 16-dimeC ₂₆	2683	-	2.8 (0.2)	4.3 (0.5)
<i>n</i> -C ₂₇	2700	12.0 (3.2)	2.8 (0.4)	3.1 (0.3)
7-,9-, 11-,13-meC ₂₇	2735	-	16.3 (5.9)	2.4 (0.6)
9-,13-dimeC ₂₇	2768	-	14.0 (2.5)	9.8 (1.6)

Table 4a.3 The composition of cuticular hydrocarbons (mean $\pm$ SD) of workers of three termite species-Continued

Hydrocarbon	ECL ^a	Relative amount (%)		
		<i>I. minor</i>	<i>C. formosanus</i>	<i>R. speratus</i>
3-meC ₂₇	2774	-	4.5 (1.7)	0.5 (0.2)
<i>n</i> -C ₂₈	2800	-	0.7 (0.2)	-
9-, 13-dimeC ₂₈	2868	-	3.5 (0.9)	-
<i>n</i> -C ₂₉	2900	1.7 (0.8)	0.6 (0.4)	-
9-,11-,13-,15-meC ₂₉	2938	-	2.3 (1.3)	-
9-,13-dimeC ₂₉	2966	1.9 (1.1)	4.4 (0.5)	-
10-,14-dimeC ₃₀	3067	1.4 (0.5)	-	-
11-meC ₃₁	3133	1.2 (0.7)	-	-
12,16-dimeC ₃₁	3170	11.9 (3.0)	-	-
9-,11-meC ₃₂	3233	0.4 (0.2)	-	-
12-,16-dimeC ₃₂	3262	2.6 (1.3)	-	-
9-,11-meC ₃₃	3334	2.7 (0.9)	-	-
3-,13-dime and/or 12-,16-dimeC ₃₃	3368	5.9 (2.2)	-	-
12-,16-dimeC ₃₄	3468	1.8 (0.3)	-	-
Unsaturated	> 3458	15.5 (3.4)	11.5 (1.6)	-

^a ECL; Equivalent chain length

4.4.4 Desiccation atmosphere

In our study, the CP values for *C. formosanus* (Meeh: $29.02 \pm 1.23 \mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$; and Haagsma: $43.09 \pm 1.77 \mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$) ranged between the values reported by Sponsler and Appel (1990) and Shelton and Grace (2003). The other subterranean termite, *R. speratus*, had CP values of $32.38 \pm 1.19 \mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$ for the Meeh formula and $43.46 \pm 1.71 \mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$ for the Haagsma formula. Between these species, the CP values were not significantly different ($P > 0.05$). Similar to other studies (Shelton and Grace, 2003; Sponsler and Appel, 1990), the CP values of these two subterranean termite species were significantly greater than those of *I. minor*. Our CP values for *I. minor* were similar to those reported by Rust et al. (1979) ($1.5\text{--}2.3 \mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$) and Haagsma ($2.5 \mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$).

For the subterranean termites, *R. speratus* lost approximately 7.38% of body water per hour up to 11 h ($Y = 7.3828x + 5.8648$; $R^2 = 0.9849$), and *C. formosanus* lost 4.65% of body water per hour up to 16 h ($Y = 4.6549x + 5.8253$; $R^2 = 0.9938$). Mortality greater than 50% was observed with %TBW loss of $53.6 \pm 2.0\%$ for *R. speratus* and $63.1 \pm 1.4\%$ for *C. formosanus* (Fig.4a.2 and Table 4a.3). In a comparison of the subterranean termites, the first mortality was recorded after 4 h ($15.00 \pm 4.28\%$; mortality = 8/60) and 6 h of exposure ($6.67 \pm 2.11\%$; mortality = 4/60) for *R. speratus* and *C. formosanus*, respectively. The lethal threshold levels for cumulative %TBW loss of *R. speratus* were $57.4 \pm 1.7\%$ (LTL₅₀) and $79.6 \pm 1.7\%$ (LTL₉₅) and for *C. formosanus*, the values were $60.4 \pm 1.3\%$ (LTL₅₀) and $80.1 \pm 1.9\%$ (LTL₉₅). The mortality reached 100% when the cumulative %TBW loss was $81.2 \pm 2.0\%$ and $83.6 \pm 0.7\%$ for *R. speratus* and *C. formosanus*, respectively. The %TBW loss for these subterranean termites increased linearly until 100% mortality.

The drywood termite *I. minor* was highly resistant to desiccation, and although the cumulative %TBW loss increased linearly throughout the 24 h experiment ($Y = 0.2574x + 0.4904$; $R^2 = 0.9917$), no significant effect on mortality was detected (Fig. 4a.2 and Table 4a.4). The first record of mortality in the *I. minor* replicates was after 18 h of exposure ($1.16 \pm 1.16\%$; mortality = 1/60) and reached $6.67 \pm 2.11\%$ (mortality = 4/60) after 24 h. The majority of *I. minor* termites survived the 24 h of desiccating conditions, and as a result, we did not obtain LTL₅₀ or LTL₉₅ values.

Table 4a.4 Power function regression coefficients (mean $\pm$ SE) for % TBW lost over 24 h for three termite species exposed to the two test atmospheres (see Fig. 4a.2)

	A	b	C	F	P	R²
<i>I. minor</i> (D)	0.415 $\pm$ 0.031	-0.018 $\pm$ 0.003	0.001 $\pm$ 8.35E-005	31929.5204	< .0001	0.9982
<i>C. formosanus</i> (D)	6.075 $\pm$ 0.324	-0.074 $\pm$ 0.032	-0.001 $\pm$ 0.001	3854.0124	< .0001	0.9991
<i>R. speratus</i> (D)	11.597 $\pm$ 0.383	-0.449 $\pm$ 0.038	0.006 $\pm$ 0.001	3261.9975	< .0001	0.9989
<i>I. minor</i> (C)	0.416 $\pm$ 0.180	0.027 $\pm$ 0.018	4.36E-005 $\pm$ 0.0005	1037.3018	< .0001	0.9966
<i>C. formosanus</i> (C)	0.673 $\pm$ 0.076	0.054 $\pm$ 0.007	-0.001 $\pm$ 0.0002	10888.7402	< .0001	0.9997
<i>R. speratus</i> (C)	0.893 $\pm$ 0.102	0.161 $\pm$ 0.01	-0.004 $\pm$ 0.0003	18528.5203	< .0001	0.9998

%TBW lost against low relative humidity and induced high CO₂ atmospheres was modeled using the polynomial cubic $y = ax + bx^2 + cx^3$.

(D) denotes a desiccated atmosphere (C) denotes a high CO₂ atmosphere

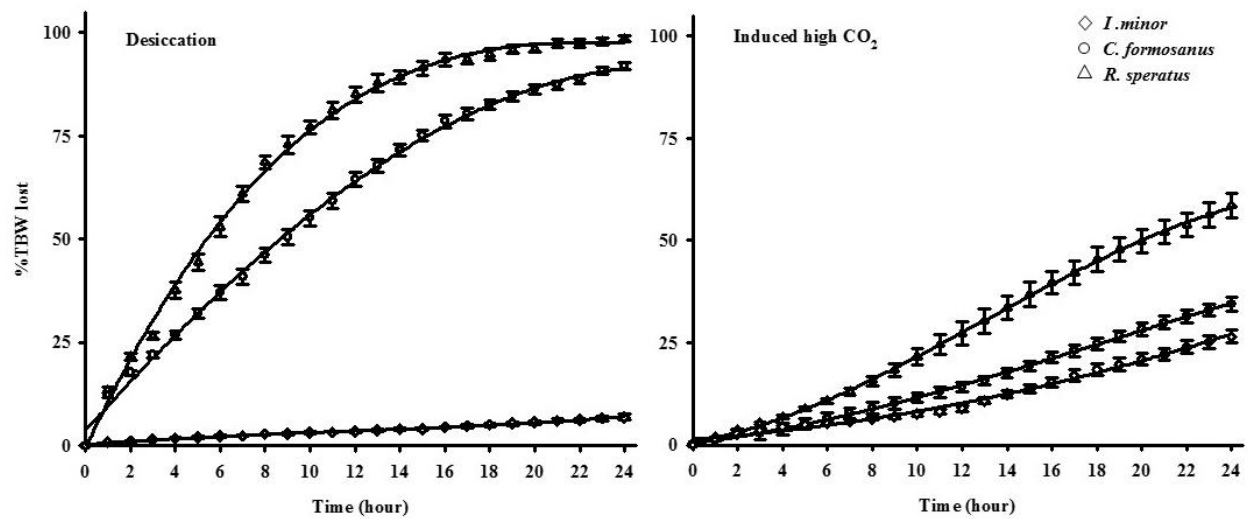


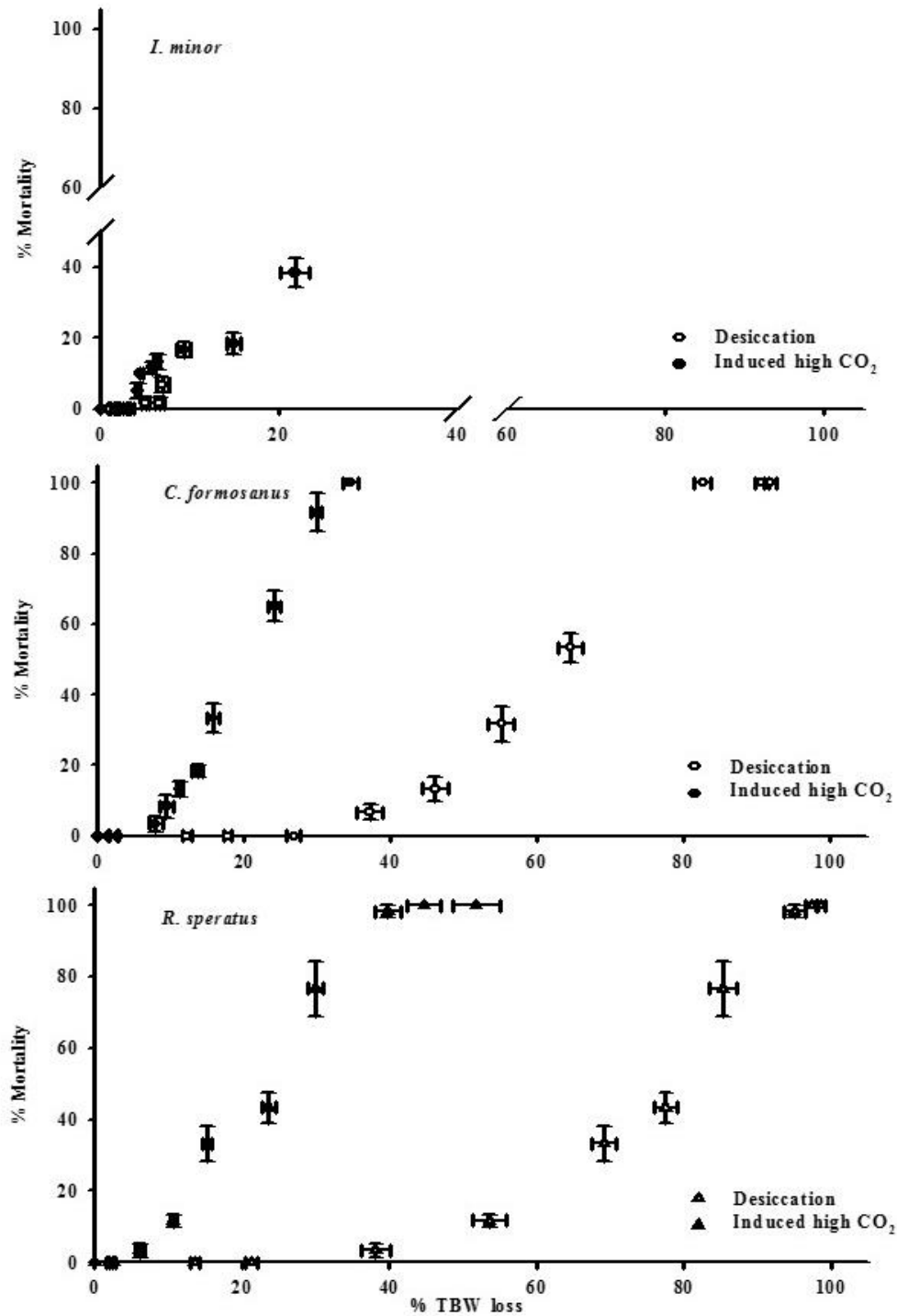
Figure 4a.2 Relationship of mean cumulative %TBW lost with time for three termite species exposed to desiccated (0–2% RH at 28°C) and induced high CO₂ (6% RH at 28 °C) atmosphere

4.4.5 Induced high carbon dioxide atmosphere

The sublimation of dry ice inside the test chamber saturated the atmosphere with CO₂ (concentration > 95%) approximately 11 min after the dry ice was placed in the CO₂ supply chamber. When the chamber was saturated, the RH was reduced and remained at 6% throughout the experiment. At CO₂ concentrations greater than 15%, direct observations of subterranean termites showed behavioral aberrations that included intense shaking, flipping, and bending of the body, losing balance, and walking backward. CO₂ narcosis/immobilization was achieved at $40.0 \pm 2.0\%$ for *R. speratus* and at $50.0 \pm 2.0\%$ for *C. formosanus*. By contrast, *I. minor* showed more resistance to high concentrations of CO₂ because we did not record any behavioral aberrations until CO₂ narcosis/immobilization ($80.0 \pm 5.0\%$). When first removed from the high CO₂ atmosphere, the termites were inactive (up to 6 h) before beginning to move. After CO₂ narcosis/immobilization, full locomotion was typically achieved within 2 h, and the maximum mortality was 10% for subterranean termites and 3% for drywood termites.

Based on the correlation between time and %TBW loss, subterranean termites exhibited significantly higher resistance to %TBW loss in the high CO₂ atmosphere than when in the desiccated atmosphere (Fig. 4a.2). The CP values for *R. speratus* calculated with Meeh's and Haagsma's formulas were $5.33 \pm 0.21 \mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$ and $7.10 \pm 0.28 \mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$, respectively. For *C. formosanus*, the CP values were $4.60 \pm 0.42 \mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$ from Meeh's formula and $6.97 \pm 0.62 \mu\text{g H}_2\text{O cm}^{-2} \text{h}^{-1} \text{mm Hg}^{-1}$ from Haagsma's formula. The CP values for *C. formosanus* remained lower than those of *R. speratus* throughout the 24 h exposure. Nevertheless, based on the correlation between mortality and %TBW loss, the susceptibility of subterranean termites increased in the induced high CO₂ conditions. For the *R. speratus* population, the mortality was greater than 75% with less than 30% TBW loss. The results were similar for *C. formosanus*, with approximately 24.2% of TBW loss (mean $\pm$ SE) resulting in $65.0 \pm 4.3\%$ mortality (Fig. 4a.3).

In contrast to the subterranean termites, *I. minor* was apparently more susceptible to mortality based on %TBW loss in the induced high CO₂ atmosphere (Figs. 4a.2 and 4a.3). Compared with the identical period in the desiccated atmosphere, the %TBW loss after 2 h increased 2.5-fold (1.1% $\rightarrow$ 2.7%). By the end of the 24 h exposure, the %TBW loss increased 2.1-fold (6.9% $\rightarrow$ 21.9%). Consequently, the mortality increased 5.7-fold (6.7% $\rightarrow$ 38.3%) after 24 h of exposure. Because of the high %TBW loss in the first 2 h, the CP values also increased significantly (Meeh: $t = -7.923$, $df = 10$, $P < 0.001$; and Haagsma: $t = -7.925$, $df = 10$, $P < 0.001$) compared with those in the desiccated atmosphere (Table 4a.1). The high mortality was likely caused by carbon dioxide asphyxiation instead of the low %TBW loss.



*Dots indicate 10 time intervals of observation (1, 2, 4, 6, 8, 10, 12, 18, 21, and 24 h)

Figure 4a.3 Relationship of mean cumulative %TBW lost with mortality% for three termite species exposed to desiccated (0–2% RH at 28°C) and induced high CO₂ (6% RH at 28 °C) atmosphere

4.5 Discussion and conclusion

In phylogeny, homologous characters are inherited from generation to generation, which is heredity (Kather and Martin, 2012). As demonstrated in numerous comparative studies of adaptations, our data show that morphological adaptations (i.e., a relatively large body size, a large mass of reserves that are metabolized by reducing body water mass, a mass of cuticular lipids, and cuticular hydrocarbons) influenced the cuticular resistance in desiccating conditions (Chown et al., 2011; Danks, 2000; Gibbs et al., 1997; Haverty et al., 1988; Howard et al., 1978; Lockey, 1988; Tucker, 1977; Woodrow et al., 2000). Compared with the two species of subterranean termites, the body size of *I. minor* was a minimum of 1.1- and a maximum of 1.8-fold larger, and a number of total lipids was a minimum of 1.7- and a maximum of 3.1-fold greater (particularly for the amount of cuticular lipids, which were a minimum of 1.8- and maximum of 2.1-fold greater), which increased the resistance to desiccation in the desiccated environment. From the analysis of the chemical taxonomy of cuticular hydrocarbons (CHC), which are often exposed to a desiccating environment in termites, *I. minor* possessed greater quantities of cuticular hydrocarbons than those of the subterranean termite species (Table 4a.2). In particular, the larger proportion of saturated cuticular hydrocarbons of *I. minor* indicated the greater the effectiveness of the cuticular lipids in preventing desiccation (Howard et al., 1978). However, the resistance to desiccation in a termite is not solely a function of one morphological factor, and many factors can increase the tolerance to desiccation.

In addition to morphological adaptations, adaptive behaviors of *I. minor*, including aggregation and reducing locomotion, were observed, which also reduce evaporative water loss. The water lost from insects through the combination of defecation and respiration is less than 20% of the TBW losses, which implies that more than 80% of the TBW loss is through cuticular transpiration (Gibbs

et al., 1997; Quinlan and Hadley, 1993; Williams and Bradley, 1998). Therefore, behavior such as aggregation increases survival by reducing water loss from a reduced surface of exposed cuticle and lower metabolic rates (Yoder et al., 1992). Similar behavior was also observed in Emperor moth caterpillars (Klok and Chown, 1999) and other drywood termites (Cabrera and Rust, 1996; Gautam and Henderson, 2011b; Minnick et al., 1973) in which the survival rates of individuals from aggregations are higher than those of single individuals. In a combination of behavioral and physiological responses to decrease the water loss, *I. minor* also reduced locomotion, which leads to a decrease in tracheal ventilation and a decrease in water loss through cuticular transpiration. By reducing locomotion, the termite also lowers the consumption of O₂ with lower metabolic rates, and therefore, the water lost from the spiracles is reduced (Cabrera and Rust, 1996). In this study, the vigorously moving subterranean termites lost %TBW at a much faster rate than the lethargic *I. minor*.

As a general rule, insects that live in arid/xeric environments have lower CP than those living in mesic/hydric environments (Eckstrand and Richardson, 1980; Kærsgaard et al., 2004; Shelton and Grace, 2003; Zachariassen, 1991) because the epidermis restricts water movement from the cuticle (Riddle, 1981). However, this relation did not apply in the induced high CO₂ atmosphere. In the high CO₂ atmosphere, the most striking difference was the %TBW loss among the termite species. Compared with the desiccating environment, in the high CO₂ atmosphere, the %TBW loss was significantly lower in the subterranean termites but was greater in the drywood termites (Fig 5a. 2). The relatively high RH (6%) that occurred with CO₂ narcosis/immobilization might have influenced the significant difference in the %TBW. Many quantitative studies on various insect species demonstrate that CO₂ destroys activity rhythms, including reducing locomotion (Badre et al., 2005; Ebadi et al., 1980; Nicolas and Sillans, 1989), by decreasing sensitivity to glutamate at

neuromuscular junctions (Nilson et al., 2006). As the concentration of CO₂ increases, the heart rate slows and eventually, heart activity is arrested without damage to the insect (Edwards and Patton, 1965; Ward, 1971). However, when a toxic threshold is exceeded, which in this test was after 12 h @ > 95% CO₂, a high CO₂ atmosphere causes sublethal or lethal effects to subterranean termites. Consequentially, a physiological response to high CO₂ concentrations is a reduction in the metabolic rate (Howarth, 1983). Thus, we hypothesized that the lower %TBW loss in the subterranean termites might be correlated with a low metabolic rate caused by CO₂ narcosis and/or asphyxia.

In contrast to this hypothesis, a low metabolic rate did not pertain to drywood termites because the %TBW loss was much higher in the high CO₂ than that in the desiccated atmosphere. One hypothesis to explain the higher %TBW losses in the high CO₂ atmosphere for *I. minor* was the absence of social aggregation, which may have strong repercussions for insects (Nicolas and Sillans, 1989) because of the increase in water loss. In this study, unlike in the desiccated environment, social aggregation was not observed during the exposure of *I. minor* to high CO₂. The relaxation of muscles provides an alternative hypothesis. We noted that groups of *I. minor* defecated on average 14–20 pellets (mean 0.91 mg ± 0.2; 1.0% of wet weight) under CO₂ narcosis. In this scenario, CO₂ initially causes an oxygen deficit, and lactic acid is produced in the muscles; then, the high lactic acid content reduces the performance of muscles such as those of the termite anus, which leads to an increase in defecation. As a final alternative, CO₂ may exert its narcotic effect directly on the respiration system by causing tonic discharging to the spiracular muscles (Mill, 1985). With an increase in the external CO₂ concentration, the ventilation rate is affected, and the permanent opening of the spiracles causes water loss to fatal levels (Bursell, 1974).

The results of this study support the hypothesis that termites evolved a variety of morphological, physiological, and behavioral adaptations that together permit survival in dry natural habitats. However, these biological adaptations were incapacitated to a degree in an atmosphere with an artificially induced high CO₂ concentration that was sufficient to eradicate drywood termites. Although the mechanism for the increased loss of body water under high CO₂ conditions was not determined in our study, for practical application, the time required for fumigation can be reduced because of the increase in the rate of body water loss from drywood termites in high CO₂ conditions.

Publication B Do northern populations of *Reticulitermes speratus* (Kolbe) possess an additional physiological capacity to cold acclimate that enhances cold tolerance during the winter?

4.1 summary

We measured and compared different carbohydrates, the total lipids, and the body water content between summer- and winter-acclimatized *Reticulitermes speratus* (Kolbe) in three latitudinal regions to determine whether northern populations possess an additional physiological capacity to acclimate to cold temperatures.

The results of supercooling points and the lower lethal temperatures of summer- and winter-acclimatized *R. speratus* workers revealed that these termites are adapted to cold climates by altering their carbohydrate metabolites (e.g., glucose and trehalose), and balanced their body water content and total lipids to improve their survivability by adapting the physiological strategies to tolerate inclement environment conditions . We observed a mean SCP of -5.1°C for summer-acclimatized *R. speratus*, which is below the observed mean lower lethal temperature; their SCP slight increase to -6.9°C with winter-acclimatized *R. speratus*. Within three contrasting locations, *R. speratus* populations in Kyoto had higher levels of carbohydrate metabolites alteration, lower supercooling points, and lower lethal temperatures. The results of this study support the null hypothesis that termite adapted the physiological strategies to tolerate cold environment. However, the geographical range of *R. speratus* is not necessarily dependent on only its physiological strategies. Instead, we conclude that it appears to rely on a combination of behavioral, physiological, and other competitive adaptations that ensure the survival of the whole colony under low, non-optimal temperatures. Additionally, the northern populations of *R. speratus* do not

possess an additional physiological capacity to cold-acclimate that enhances cold tolerance during the winter.

4.2 Introduction

In general, insects living at higher latitudes tend to be more tolerant to low temperatures than their counterparts because temperature variation increases with latitude (Addo-Bediako et al., 2000; Sinclair et al., 2003a; Sunday et al., 2012) This hypothesis may reflect the evolution of a suite of biological adaptations in higher latitude organisms to cope with temperature variation. This hypothesis has also led to many studies seeking to understand the mechanisms of thermal tolerance of insects (Bale, 2002; Chown et al., 2004; Danks, 2000; Singh and Khan, 2009; Storey and Storey, 1991; Teets and Denlinger, 2013) including termites (Cabrera and Kamble, 2004; Clarke et al., 2013; Lacey et al., 2010; Sponsler and Appel, 1991).

A classic example of a termite behavioral trait is habitat exploration. Hu and Appel (2004) reported that *Reticulitermes* spp. were able to adjust their minimum critical temperature to survive the seasonal change in soil temperature. Furthermore, their ability to excavate enables them to relocate their nest to deeper ground, where soil temperature is buffered from the climate (Clarke et al., 2013; Hu and Song, 2007; Strack and Myles, 1997). In natural settings, the habitat for termites is often underneath stumps and large pieces of decayed logs, (Grace, 1996) whereas in urban areas they can be found near heated structures and in sewer systems (Myles and Grace, 1991; Scaduto et al., 2012). In contrast to studies on the behavioral strategies of termites, much research has been published on the physiological adaptations and changes in termites in response to a gradual or abrupt decrease in temperature (Cabrera and Kamble, 2004; Davis and Kamble, 1994; Hu and Appel, 2004; Sponsler and Appel, 1991; Strack and Myles, 1997; Woodrow et al., 2000). Lacey et al. (2010) recently reviewed the research on the physiological strategies of the order

Isoptera (termites) and revealed that two dampwood termites, *Porotermes adamsoni* Froggatt and *Stolotermes victoriensis* Hill, enhanced their cold tolerance by synthesizing (i.e., increasing) trehalose and unsaturated lipids which function as cryoprotectants. A disaccharide and non-reducing sugar, trehalose, has been found to be a cryoprotectant in many insect species (Fuller, 2004; Rudolph and Crowe, 1985; Storey and Storey, 1991; Thompson, 2003) that acts by stabilizing insect membranes during hypertonic exposure. Trehalose usually constitutes 1–2% of the sugars in haemolymph insects (Becker et al., 1996) including termites (Itakura et al., 2006; Lacey et al., 2010). The biosynthesis of trehalose (Arrese and Soulages, 2010; Candy, 1985; Nomura and Ishikawa, 2001) uses glycogen reserves and occurs when insects are under temperature stress. Cold induction can also affect other metabolites that serve as cryoprotectants, and cold tolerant insects can also acclimate to cold temperatures by altering their lipid content (Overgaard et al., 2007; Roy et al., 1991) and glucose (Costanzo et al., 1993; Storey and Storey, 1985) to increase their survivability during slower and less abrupt seasonal changes in temperature. Although above researches show that many physiological adaptations may allow termites to survive at cold temperatures, we are unaware of research demonstrating whether physiological adaptations play a key role in limiting their range of distribution

Reticulitermes speratus (Kolbe) is a well-known native termite species that is considered to be an urban pest in Japan due to its wide range of distribution throughout the tropical rainforest (Okinawa) and humid continental (Hokkaido) climate zones. Based on its taxonomy, *R. speratus* can be subdivided into five subspecies: *R. speratus speratus*, *R. speratus leptolabralis*, *R. speratus kyushuensis*, *R. speratus okinawanus*, and *R. speratus yaeyamanus* (Morimoto, 1968). Of these subspecies, *R. speratus* and *R. speratus kyushuensis* are the most important in economic terms in Japan. Several Japanese studies (Ikehara, 1966; Matsumoto, 1983; Morimoto, 1975) reported that

the northern range limit of *R. speratus* corresponds with the region where the average January temperature does not dip below -4.0°C . However, a well-established *R. speratus* colony is present in Asashigawa, Hokkaido (JWPA, 2013) where the mean daily temperature and the mean minimum temperature in January are -7.9 and -12.7°C , respectively (JMA, 2014). In the same genus, *R. flavipes* (Kollar) has been observed inhabiting the southernmost tip of Ontario, Canada which is well north of its native range in North America (Raffoul et al., 2011). The distribution of *Reticulitermes* spp. would indicate that *R. speratus* – similar to other termite species – can survive winter conditions by adapting to the cold using one or more biological adaptation. (Cabrera and Kamble, 2001; Davis and Kamble, 1994; Lacey et al., 2010)

The objectives of this study were 1) to measure the supercooling points and the lower lethal temperatures of summer-acclimatized *R. speratus* (as controls) and winter-acclimatized *R. speratus* from three contrasting locations and 2) to compare the physiological, biochemical traits – including the carbohydrate metabolites (glycogen, glucose, and trehalose), body water content, and total lipid content – in termites to determine whether *R. speratus* has evolved physiological adaptations to cope with temperature variation. Finally, we aimed to project the potential distribution of *R. speratus* across Japan and worldwide.

4.3 Materials and methods

4.3.1 Collection of termites

Termites from three colonies of *R. speratus* were collected from each of three regions distributed across a latitudinal gradient in summer and winter: 1) Sapporo City, Hokkaido, northern Japan (43.071°N , 141.341°E : within a humid continental climate zone based on the Köpper-Geiger climate classification); 2) Uji City, Kyoto, central Japan (34.909°N , 135.797°E : within a humid subtropical climate zone); and 3) Hioki City, Kagoshima, southern Japan (31.513°N , 130.331°E :

within a humid subtropical climate zone). Termites were collected from colonies in tree stumps, fallen logs and branches in the forest at the sampling sites in each region in early summer (end of July) and winter (end of November). Collected termites were packed in plastic containers with ground soil from the different areas and sent to the laboratory. During two days of travel, all samples were kept within a temperature range similar to that of their natural environment (3.0 °C). After the arrival of the samples, the termites from each region were immediately removed from the transported decayed logs and branches for determination of supercooling points, lower lethal temperatures, carbohydrate metabolites, total lipids and body water content.

4.3.2 Bioassays

For the analysis of the supercooling points (SCPs) of the termites, we prepared five replicates (10 termites/replicate) for each colony from the three locations for each season (five replicates per colony x three colonies x three regions x two seasons = 90 replicates). To determine the SCPs, we used contact thermocouple thermometry, as described by Hanson and Venette (2013), but with a slight modification. A 24-gauge copper-constantan thermocouple was formed into a spiral and placed in a 2 mL syringe tube; termite specimens were placed in gelatin capsules (110 x 80 x 200 mm) and attached to the coiled thermocouple. The thermocouple wire leading out of the syringe was connected to a computer via a thermocouple interface, and SoftThermo E830 software (Technol Seven, Tokyo, Japan) was used for the data analysis. The thermocouples with termites were cooled to -20.0 °C at a constant of -1.0 °C per minute inside an incubator (Eyela KCL-2000A, Tokyo, Japan). The SCP was recorded from the temperature plots as the lowest temperature reached before the release of latent heat of fusion.

For the analysis of the lower lethal temperatures (LLTs) of the termites, the same experimental design for determination of the SCPs (Rust et al., 1997) was used in which there was a total of 90

replicates (10 termites/replicate). For each replicate, termites were placed in a glass Petri dish over a moistened WhatmanTM number 2 filter paper (diameter 5.5 cm with 0.5 mL of distilled water) and then placed in an Eyela KCL-2000A incubator at natural temperature conditions for *R. speratus*. Both the summer and winter samples underwent a cold-shock process in which termites were exposed to a series of temperature settings (−4, −5, −6, −7, −8, −9, −10, −11.0, −12.0 and −13.0 °C). After the designated temperature had been reached (at a rate of −1 °C/min), it was maintained for 30 min. After the 30 min period of cold-shock, the Petri dishes were removed and left for 2 h at room temperature for termites to either recover from chill-coma or die; they were then left overnight in a conditioning chamber (at 23 °C with a relative humidity of 75%). Next, the indices of mortality were determined by separating and counting the live and dead termites after overnight exposure. The LLT₅₀ and the LLT₉₅ were calculated using the mortality data once mortality had reached 100%.

4.3.3 Measurement of carbohydrate metabolites

For the analysis of carbohydrate metabolites 36 replicates (300 termites/replicate) were prepared (two replicates per colony x three colonies x three regions x two seasons = 36 replicates). For each replicate, termites were placed in glass Petri dishes over WhatmanTM number 2 filter paper and then transferred to an oven (Eyela NDO-600ND, Rikakikai, Tokyo, Japan) for drying at 100 °C for 10 min followed by additional drying at 60 °C for 48 h. The procedure described by Itakura et al. (2006) was used in sample preparation. Each sample was homogenized by combining 100 mg of dried termites with 3.0 mL and placing it in a Potter-Elvehjem ice-bath homogenizer (Sigma-Aldrich, St. Louis, MO, USA). Homogenized samples were divided into three aliquots for measurement of glycogen, glucose, and trehalose content. Each aliquot was centrifuged at 15,000 *g* for 10 min (Kubota 3500, Kubota, Tokyo, Japan).

For the glycogen analysis, 1.0 mL of 0.3 mol of trichloroacetic acid (TCA) was added to 1.0 mL of the supernatant, and the mixture was refrigerated (4 °C) for 2 h. It was then centrifuged for 20 min at 15,000 g. Next, 2.0 mL of the supernatant was removed and placed in a 15 mL centrifuge tube, to which 8 mL of ethanol was added. The mixture was kept in a refrigerator (4 °C) overnight. To separate the glycogen pellet and the supernatant, 10 mL of the glycogen solution was centrifuged at 9,500 g for 20 min (Tomy SRX-201, Tomy Seiko, Tokyo, Japan). After removing the supernatant, 0.5 mL of 0.68 mol of hydrochloric acid (HCl) was added to the tube containing the glycogen pellet. The tube was sealed and heated for 2.5 h in a heat bath (100 °C). Once the mixture had cooled, 0.5 mL of 0.68 mol of sodium hydroxide (NaOH) was added for neutralization. To measure the glucose content, 0.6 mL of supernatant was transferred to a new centrifuged tube (1.5 mL) and kept in a refrigerator (4 °C) until analysis. For the trehalose analysis, 1.0 mL of 1 mol of HCl was added to 1.0 mL of supernatant, and the tube was then sealed and heated for 1 h in a heat bath (100 °C). Once the mixture had cooled down, 1.0 mL of 1 mol of NaOH was added for neutralization. Samples were kept in a refrigerator (4 °C) until analysis.

The methods described by Nelson (1944) and Somogyi (1952) were applied to measure the amounts of glucose in the supernatants and the glycogen and trehalose in the hydrolysates of the termite homogenate. First, 0.5 mL of supernatant was mixed with 0.5 mL of alkaline copper reagent (Somogyi) and heated for 20 min. Arsenomolybdate (Nelson) was added after the mixture had cooled down. The resultant solutions were diluted with 11 mL of distilled water before they were transferred to a 10-mm cell for recording the absorbance at 660 nm using a spectrophotometer (UV2450, Shimadzu, Kyoto, Japan).

4.3.4 Measurement of total body lipid and body water content

A total of 36 replicates (two replicates per colony x three colonies x three regions x two seasons) were prepared for the measurement of termite body water content and the total lipid content. For each replicate, termites were weighed to the nearest 0.01 mg using a semi-micro analytical balance (A&D-GH252, Tokyo, Japan). They were then placed in an oven for drying at 100 °C for 10 min, followed by an additional drying at 60 °C for 48 h after which the total body water content could be determined. Next, the dried termites were used for lipid extraction by simply using a non-polar solvent. Termites were pre-weighed and then their heads and abdomens were severed and separated for testing. Each portion (e.g., abdomens) was placed in a glass vial (15 mL) to which 2 mL of chloroform was added. The vials were left for 48 h to allow lipid extraction to occur. The exoskeleton in the extraction solution was removed by filtration through an Omnipore membrane filter (0.2 µm JG type, Darmstadt, Germany). Next, the extraction was dried again at 60 °C for 48 h to obtain the dry weight of the exoskeleton without the total lipids. The percentage of the total lipids was calculated as $([\text{dry weight before extraction} - \text{dry weight after extraction}] / \text{dry weight before extraction}) \times 100$.

4.3.5 Statistical analysis

For the determination of the lethal temperature that induced a 50% and 95% mortality of termites, a probit analysis was conducted to correlate the change in mortality with the exposure time. All statistical analyses were conducted using SPSS ver. 18 (SPSS, 2009). Analysis of covariance (ANCOVA) was used to compare carbohydrate, total lipids, and supercooling point (dependent variables) data for termites collected on each season across the region (independent variables) over dry weight (covariate) and body water content (independent variables) over fresh weight(covariate). To determine possible differentiation between populations, the data were

compared and separated by Tukey's post-hoc test and general linear model with region and season. Significance levels were set at $P \leq 0.05$.

4.4 Results

4.4.1 Bioassay

4.4.1.1 Supercooling point (SCP)

The SCP of *R. speratus* worker tended to decrease with changes of season. We recorded significantly lower SCP along winter-acclimatized termites than summer-acclimatized termite ($F_{(2,41)} = 6.052$, $P = 0.005$; Table 4b.1). The largest reduction in the mean SCP between the two termite acclimatization groups was recorded in Kyoto (from -5.2 ± 0.13 °C to -7.3 ± 0.19 °C), followed by Hokkaido (from -5.1 ± 0.10 °C to -6.8 ± 0.14 °C), and finally Kagoshima (from -5.0 ± 0.12 °C to -6.6 ± 0.15 °C).

4.4.1.2 Lower lethal temperatures (LLT₅₀ and LLT₉₅)

When the summer- and winter-acclimatized *R. speratus* were exposed to the cold-shock process, all individuals entered into a chill coma. However, as long as the temperature did not dip below their lethal level, all individuals revived briefly after rewarming. For the summer-acclimatized *R. speratus*, all individuals exposed to -4.0 °C recovered from a chill coma after rewarming, although frozen individuals were observed as the temperature (termite experienced) decreased. At the -5.0 °C setting, the first cases of mortality (less than 5%) were recorded for termites from all three locations; however, no significant difference was found in the rate of mortality across locations ($F = 2.0222$, $df = 44$, $P = 0.1450$). With extended temperature exposure at -7.0 °C, we observed a sudden surge in mortality ($> 65\%$); therefore, we could determine the LLT₅₀ (Table 4b.1). At this temperature there was no significant difference in the rate of mortality among the contrasting locations ($F = 0.9275$, $df = 44$, $P = 0.9275$). The LLT₉₅ for *R. speratus* was recorded.

Table 4b.1 The results of the analysis of 1) carbohydrate metabolites, 2) the supercooling point (SCP), and 3) the lower lethal temperatures (LLT₅₀ and LLT₉₅) in summer- and winter-acclimatized *Reticulitermes speratus* (Kolbe) from three different colonies

Table location	H ₂ O (% of wet wt)	Dry weight (mg/termite)	Total lipid (% of dry wt)	Carbohydrate metabolites			SCP (°C)	LLT ₅₀ * (°C)	LLT ₉₅ * (°C)
				Glucose (% of dry wt)	Trehalose (% of dry wt)	Glycogen (% of dry wt)			
Kagoshima (Summer)	83.0 ± 0.4 ^{a(a)}	0.405 ± 0.007 ^{c(a)}	22.4 ± 0.6 ^{a(a)}	2.09 ± 0.03 ^{a(b)}	1.88 ± 0.06 ^{a(a)}	1.14 ± 0.04 ^{ab(a)}	−5.0 ± 0.1 ^{a(b)}	−6.7 −7.0	−9.8 −11.5
Kyoto (Summer)	82.1 ± 0.3 ^{a(a)}	0.441 ± 0.010 ^{b(a)}	22.9 ± 0.9 ^{a(a)}	2.13 ± 0.13 ^{a(b)}	2.10 ± 0.09 ^{a(b)}	1.42 ± 0.02 ^{a(b)}	−5.2 ± 0.2 ^{a(b)}	−6.8 −7.4	−9.9 −11.8
Hokkaido (Summer)	75.1 ± 0.3 ^{b(a)}	0.585 ± 0.012 ^{a(a)}	17.7 ± 0.6 ^{b(a)}	1.60 ± 0.07 ^{b(b)}	1.41 ± 0.08 ^{b(b)}	0.95 ± 0.06 ^{a(a)}	−5.1 ± 0.1 ^{a(b)}	−6.7 −7.0	−9.7 −11.8
Kagoshima (Winter)	80.7 ± 0.3 ^{ab(b)}	0.393 ± 0.012 ^{b(a)}	21.6 ± 0.4 ^{ab(a)}	2.24 ± 0.04 ^{a(a)}	2.44 ± 0.20 ^{b(b)}	1.16 ± 0.10 ^{ab(a)}	−6.6 ± 0.2 ^{b(a)}	−7.4 −8.1	< −11.1
Kyoto (Winter)	78.3 ± 0.3 ^{a(b)}	0.435 ± 0.010 ^{b(a)}	24.5 ± 1.0 ^{a(a)}	2.46 ± 0.05 ^{a(a)}	3.01 ± 0.08 ^{a(a)}	1.53 ± 0.11 ^{a(a)}	−7.3 ± 0.2 ^{a(a)}	−7.7 −8.5	< −11.1
Hokkaido (Winter)	73.6 ± 0.5 ^{b(b)}	0.578 ± 0.013 ^{a(a)}	19.1 ± 1.0 ^{b(a)}	2.33 ± 0.09 ^{a(a)}	2.91 ± 0.11 ^{ab(a)}	1.03 ± 0.05 ^{b(b)}	−6.8 ± 0.1 ^{b(a)}	−7.6 −8.2	< −10.8

^a Significant difference between regions indicated by different letters ($P < 0.05$, ANCOVA)

^b Significant difference between seasons indicated by different letter in parentheses ($P < 0.05$, Post Hoc test)

^c Minimum low lethal temperature (Upper section), Maximum low lethal temperature (Lower section)

at approximately $-11.0\text{ }^{\circ}\text{C}$ (Table 5b.1), at which there was no significant difference in the rate of mortality between the different locations ($F = 1.2923$, $df = 44$, $P = 0.2853$). Regarding the winter-acclimatized *R. speratus*, the low temperatures made them slightly more cold tolerant than the summer-acclimatized *R. speratus*. The LLT_{50} and LLT_{95} of the winter-acclimatized *R. speratus* were recorded at approximately $-8.0\text{ }^{\circ}\text{C}$ and $-12.0\text{ }^{\circ}\text{C}$, respectively, and there were no significant differences in the rate of mortality at these temperatures between locations (LLT_{50} : $F = 0.2423$, $df = 44$, $P = 0.7859$; LLT_{95} : $F = 2.2750$, $df = 44$, $P = 0.1153$).

4.4.2 Carbohydrate metabolites

There was a significant difference in the mean of the dried weight of the *R. speratus* workers between the three locations (summer-acclimatized: $F_{(2,15)} = 93.115$, $P < 0.0001$, Winter-acclimatized: $F_{(2,15)} = 34.885$, $P < 0.0001$), although significant differences was detected among location, the season was not related to change of dry weight of the *R. speratus* workers ($F_{(2,41)} = 17.711$, $P < 0.0001$) (Table 4b.1).

In summer, there was a significantly higher percentage of carbohydrate metabolites in termites from the humid subtropical climate zone (Kagoshima and Kyoto) than termites from the humid continental climate (Hokkaido) (Glucose: $F_{(2,15)} = 9.352$, $P = 0.002$; Trehalose: $F_{(2,15)} = 21.964$, $P < 0.0001$; Glycogen: $F_{(2,15)} = 9.29$, $P = 0.002$). In contrast to summer, we recorded significantly higher increase of carbohydrate metabolites in termites from the humid continental climate. Mean of trehalose (a known cryoprotectant of dampwood termites) was increased approximately 106% ($1.41 \rightarrow 2.91\%$ of dry wt) compared to summer population. A similar trend was also found for another low molecular mass carbohydrate, glucose (46% increase), for the winter-acclimatized *R. speratus* from Hokkaido (Table 4b.1). In contrast to the increase of trehalose and glucose of *R.*

speratus from Hokkaido, there was no significant difference in glucose among the location ($F_{(2,15)} = 3.219$, $P = 0.069$), and narrow a trehalose gap (Trehalose: $F_{(2,15)} = 4.760$, $P = 0.025$) (Fig. 4b.1).

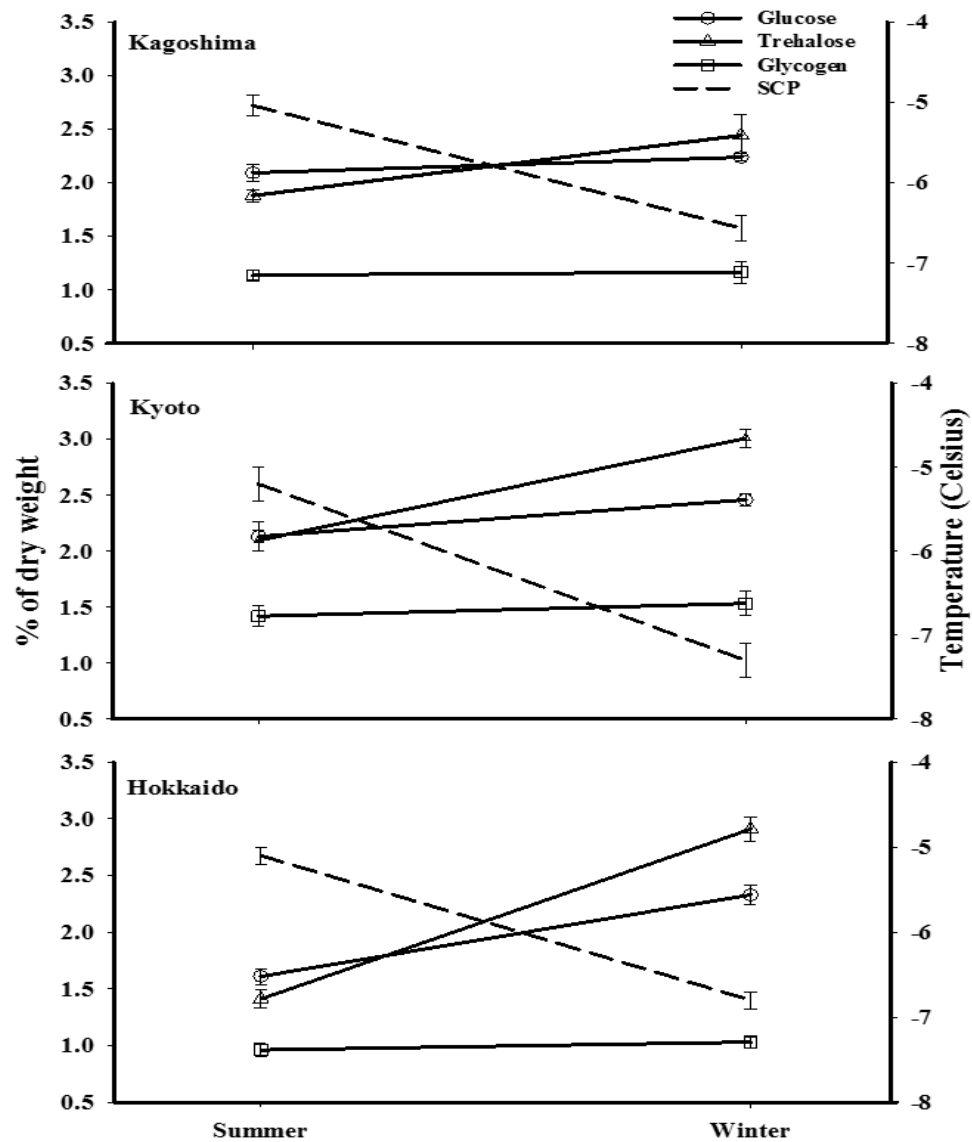


Figure 4b.1 The supercooling point (SCP) and carbohydrate metabolites during low temperature acclimation (cold-shock process) in *Reticulitermes speratus* (Kolbe) workers for three locations in Japan

4.4.3 Total body lipid and body water content

Total body lipid was significantly related to temperature. We recorded that *R. speratus* from the humid subtropical climate zone (Kagoshima and Kyoto) have a significantly higher body water content than those from the humid continental climate in both season (Summer: $F_{(2,15)} = 17.059$, $P < 0.001$; Winter: $F_{(2,15)} = 5.734$, $P = 0.015$) (Table 4b.2). In regards to acclimation to low temperature, *R. speratus* from all three locations were able to alter their body water content depending on their surroundings ($F_{(2,14)} = 11.352$, $P = 0.001$).

Balancing body water and total lipid content is known to improve their survivability in cold climates, hence, as distinct as the seasons are, it plays a big factor in the alteration of total lipid content (Fig 4b.2). These results in Table 4.1b show that total lipid content depends on the temperature condition of winter. Total lipid content in winter-acclimatized was higher than summer-acclimatized *R. speratus* from both Kyoto and Hokkaido ($F_{(2,14)} = 4.313$, $P = 0.035$). Meanwhile, less or indistinct season in Kagoshima, *R.speratus* became less stimulate to alter the lipid content in their body.

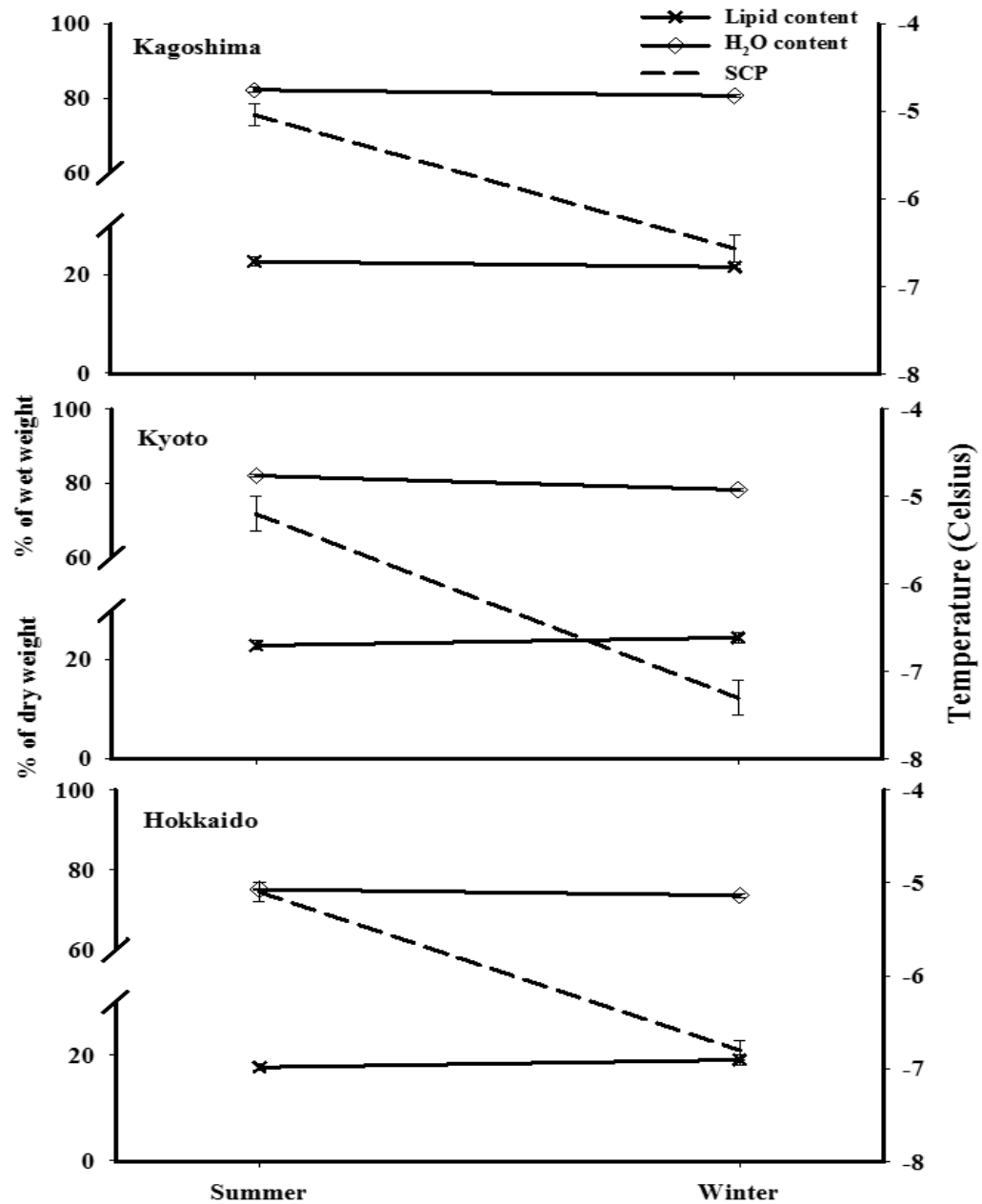


Figure 4b.2 The supercooling point (SCP), body water content and total lipid content from low temperature acclimation (cold-shock process) in *Reticulitermes speratus* (Kolbe) workers for three locations in Japan

4.5 Discussion

In this study, we measured the supercooling points and lower lethal temperatures of three colonies of summer- and winter-acclimated *R. speratus* from three contrasting locations. The supercooling

points and the lower lethal temperatures observed here reveal that these termites are adapted to their respective climates. We found that termites used the tested carbohydrate metabolites (e.g., glucose and trehalose) and balanced their body water content and total lipids to improve their survivability in cold climates. Many studies have also reported that carbohydrate metabolites in insects fluctuate dramatically in response to cold induction (Denlinger and Lee Jr, 2010; Lacey et al., 2010; Storey and Storey, 1988). It would be premature to conclude that the distribution of *R. speratus* in Japan is due to their adaptation of physiological mechanisms to cold acclimate which enhances their cold tolerance. If we assume that *R. speratus* are adapted to cold acclimate and use carbohydrate metabolites as cryoprotectants, then the *R. speratus* populations in Hokkaido, where habitat is more unsuitable for the species, should have higher levels of carbohydrate metabolites and a higher thermal resistance. However, contrary to our expectations, the results showed that *R. speratus* populations in Kyoto had altered higher carbohydrate metabolites, lower supercooling points, and lower lethal temperatures. The reason for the discrepancies in carbohydrate metabolites levels and the overall bioassay results may be related to the forest ecosystems found within the different regions and their distinct plant communities, the presence of fungi, nutrient availability, and climatic conditions.

Subterranean termites with a nutrient-poor food source consumed less wood than those with a nutrient-rich food source (Wallace and Judd, 2010). Although *R. speratus* was collected from decaying wood at three locations, the woody species making up the decaying wood were different at each location. The abundant decayed branches and logs (with a low to moderate degree of decay by white-rot fungi) are the nesting sites and food source for *R. speratus* on the moss covered forest floor of Kagoshima, and the grass and weed covered forest floor of Kyoto. Regarding the nesting sites, *R. speratus* preferred the sapwood of Japanese black pine (*Pinus thunbergii*) in both seasons.

Meanwhile, the termites from Hokkaido were collected from deciduous woodland, whose dominant tree is a species of chestnut (*Castanea crenata*). The collection sites at Hokkaido were surrounded by grassland, whereas dried leaves covered the woodland floor. The preferred habitat of the termites here is the heavily decayed heartwood of chestnut logs which receive plenty of sunlight. Studies indicate that the woody species, the wood moisture content, the type and amount of wood extracts, the proportion of heartwood to sapwood, and the species of fungus and their abundance affect the activity and survival rate of *Reticulitermes* spp. (Getty and Haverty, 1998; Nakayama et al., 2005; Ripa et al., 2002; Smythe and Carter, 1970). Most laboratory studies show that *Reticulitermes* spp. Prefer softwood over hardwood, with softwood being less dense and therefore requiring less energy to consume (Peralta et al., 2004). Termite workers break down wood with their saliva, thus gaining nutrients. By assimilating nutrients, the termites increase their amount of carbohydrate metabolites, which are used as energy and stored (Becker et al., 1996). Many studies have linked high cellulose consumption to high levels of carbohydrate metabolites (Judd and Corbin, 2009), several types of sugars, uric acid (Swoboda et al., 2004; Waller and Curtis, 2003), and ions (Botch et al., 2010). Also important to termite acquisition of different carbohydrate metabolites is the interaction of fungi with their environment. Subterranean termites often prefer wood decayed by fungi because this increases the availability of nitrogen and other nutrients in the wood by increasing the cellulose metabolic activity (Waller et al., 1987). Each fungus species breaks down wood differently and leaves different hemicellulose and sugars intact during the process of digestion (Curling et al., 2002; Wallace and Judd, 2010). Subterranean termites often prefer dead wood with brown-rot fungi. However, consumption may increase in response to nutrient-poor wood (Lenz et al., 1991; Suarez and Thorne, 2000).

Nutrient availability in the soil is another abiotic factor that influences the activity of subterranean termites. It has been shown that subterranean termite activity increases with an increase in the content of soil organic matter (Semhi et al., 2008). Many studies have shown that different organic materials can affect food selection in subterranean termites, for example, amino acids (Castillo et al., 2013; Chen and Henderson, 1996), sugars (Saran and Rust, 2005; Waller and Curtis, 2003), phosphorous, calcium (Botch et al., 2010), carbon dioxide (Bernklau et al., 2005), and nitrogen (Curtis and Waller, 1998; Gautam and Henderson, 2011a). However, our current understanding of these relationships is incomplete, as is the relative impact of soil nutrients on carbohydrate metabolite levels in *R. speratus*.

The discrepancy between the supercooling points and the lower lethal temperatures in *R. speratus* between the three locations might be related to climate. As ectotherms with a small body size, termites easily lose body heat to their environment; therefore, lower temperatures are one of the greatest challenges to survivability for *R. speratus*. *Reticulitermes speratus* is known to reduce its activity significantly at approximately 6 °C and become vigorously active at approximately 12 °C (Ikehara, 1966). The optimal temperature for the species, when its feeding activity is at a maximum, is 25-30 °C (Nakayama et al., 2004). Therefore, the longer termites were exposed to temperatures within this optimal range, the more active they became and the more they were able to forage and to assimilate carbohydrates for use as energy and reserves (Becker et al., 1996). Consequently, we can assume that due to a shorter season with optimal temperatures, *R. speratus* in Hokkaido may experience a limited window for foraging relative to the humid subtropical climate zone, which may act to limit their assimilation of carbohydrate metabolites which could affect their cold tolerance.

Contrasting opinions exist regarding whether *Reticulitermes* spp. are freezing tolerant or freezing avoidant. Davis and Kamble (1994) suggested that *R. flavipes* slightly increased its SCP during the winter, which has also been observed in a freezing tolerant insects. In contrast, Clarke et al. (2013) suggested that *R. flavipes* are freezing avoidant because these insects did not survive corporal ice formation under sub-zero temperatures. Based on our study, we observed a mean SCP of -5.1°C , which is below the observed mean lower lethal temperature; there was a slight increase in the SCP (mean -6.9°C) with acclimation to low temperatures, which suggests that *R. speratus* is freezing tolerant. Before arriving at a conclusion, there is one aspect that should be considered: how often is *R. speratus* exposed to a temperature of -6.9°C ? Although the coldest winter temperature ever recorded in Sapporo, Hokkaido (43°N) was -14.3°C (JMA, 2014), the difference between the soil temperature (0.1 m deep) and the air temperature is quite significant due to the temperature buffer provided by the soil and a thick snow cover; in fact, one study indicated that soil was frozen for a mean 15 days per year at a depth of 0.1 m whereas snow cover was present for a mean 120 days per year (Yazaki et al., 2013). Furthermore, termites adjust their low corporal temperature to seasonal changes in soil temperature (Hu and Appel, 2004) and can relocate to the soil where the temperature remains above freezing (Lee Jr, 2010; Storey and Storey, 1991). *Reticulitermes flavipes* (Kollar) is another species whose distribution includes the northernmost portion of the United States and is recognized as an urban pest in southern Ontario, Canada. The species is present at the Point Pelee National Park in southwestern Ontario, Canada (41.9642°N , 82.5178°E), which is located at a latitude similar to that of the collection site in Hokkaido (43.071°N , 141.341°E). In light of the study results, the discrepancy in the lower lethal temperatures and the supercooling points in relation to the distribution of the species, might be related to behavioral adaptations such as the burrowing behavior of *R. flavipes*, which can burrow

a tunnel as deep as 1 m to avoid freezing temperatures (Cabrera and Kamble, 2001; Sponsler and Appel, 1991; Strack and Myles, 1997). This adaptive behavior may be similar to that of *R. speratus* and may function as a primary survival mechanism, whereas physiological adaptations are additional mechanisms which ensure the survival of the entire colony survival under low, non-optimal temperatures.

The objectives of this study were to identify the variables related to cold tolerance in *R. speratus* and to predict its potential distribution and limits to that distribution in Japan. A key conclusion of this study is that carbohydrate metabolites (glucose, trehalose and balancing of total body water and total lipids) accumulate in *R. speratus* with a substantial decrease in temperature, with the effect being a slight enhancement in the cold tolerance of the species in relation to the three contrasting locations. Therefore, the geographical range of *R. speratus* does not necessarily depend on its physiological capacity to acclimate to low temperatures. Instead, it appears to be dependent on a combination of behavioral, physiological, and other competitive adaptations that ensure the whole colony survival under cold, non-optimal temperatures.

CHAPTER 5: Investigation of thermal conductive properties of structural timbers at low temperature region using solid carbon dioxide as a chilling agent

This chapter has been published as below:

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5.1 Summary

Knowledge of the thermal conductive properties of wood at low temperatures aids the development and optimization of the application of a lethal cold temperature treatment for drywood termites. This present study investigates the relationship between the thermal conductive properties of wood at low temperatures and important factors, namely, the microstructural and anisotropy of wood using two types of solid carbon dioxide as a chilling agent.

The results indicate that the thermal conductive properties of wood at low temperatures are mainly affected by the composition and morphological properties of wood (i.e., density/ratio of earlywood and latewood, the proportion of heartwood and sapwood, the pattern of growth ring). In particular, thermal conductive properties are mainly dependent on the pattern of the growth ring itself at low temperatures.

5.2 Introduction

Drywood termites may be eradicated by a lethal cold temperature treatment as an alternative to the use of pesticides and fumigants. The first investigation on using a lethal cold temperature treatment for drywood termite control was a study applying liquid nitrogen into wall voids to create temperatures below the critical thermal minimum of drywood termites (Forbes and Ebeling, 1986).

Thereafter, several other studies have reported the high efficacy of lethal cold temperature treatments under laboratory and field conditions (Lewis and Haverty, 1996; Rust et al., 1995a); however, none of these studies investigated the relationship between the thermal conductive properties of wood at low temperature and the various factors affecting these properties. Knowledge of the thermal conductive properties of wood at low temperatures can aid the development and optimization of the application of a lethal cold temperature treatment for drywood termites.

High interest of energy performance (Bouguerra et al., 2001; Lagüela et al., 2015; Sonderegger et al., 2011) and fire resistance (Clancy, 2001) of wooden structure buildings led to measurements of the thermal conductive properties of wood in past decades. Due to previous research interest, most of the measurements of the thermal conductive properties of wood were made at high temperatures. Thermal conductive properties can be categorized into three groups: thermal conductivity, thermal diffusivity, and specific heat capacity (Bouguerra et al., 2001; Steinhagen, 1977), which are the most relevant parameters for simulating the thermal behavior of wood (Sonderegger et al., 2011). The thermal conductive properties of wood are dependent on some factors. Some of the more important factors are density, porosity, and anisotropy (Mauranen et al., 2015; Suleiman et al., 1999). There is general agreement that the thermal conductive properties of wood are mainly dependent upon the cell components which are aggregated into tissue-independent phases (Dupleix et al., 2013; Eitelberger and Hofstetter, 2011). Eventually, more cell components exist as the density of wood increases. The thermal conductivity of the cell components in wood has been described in detail by many other researchers. The major cell components of wood are cellulose, and hemicellulose, which usually accounts for 65–75% of the wood dry weight and 18–35% of the lignin (Missio et al., 2015), and they are generally more

thermally conductive than air (Asako et al., 2002; Dunlap, 1914; Yu et al., 2011). Meanwhile, porosity is another obstacle to the thermal conduction of wood, due to the low thermal conductivity property of air, which fills the cellular substance with voids before the fiber saturation points. The anisotropy of the structure, a key characteristic of wood, and physical properties have been extensively discussed under low thermal properties by several researchers. Wood is a lignocellulosic material mainly comprising long-chain linear polymers (cellulose, hemicelluloses, and lignin), minor amounts of extractives and inorganic compounds (Missio et al., 2015). These components are combined and mainly aligned with the longitudinal axis of the cell. Given the anisotropic nature of wood, thermal conductivity is approximately 2-3 times higher in the longitudinal direction than in the transversal (Gu and Zink-Sharp, 2005; Kollmann and Côte Jr, 1968; Wagner et al., 1994), but it is not significantly different between the radial and tangential directions (Simpson and TenWolde, 1999; Sonderegger et al., 2011). All three parameters are strongly influenced above the fiber saturation point (FSP) and can vary by as much as 100% depending on the moisture content (Ragland et al., 1991); however, the moisture content of timber in service is well below the FSP. Therefore, it does not correspond to the notable change in the thermal conductive properties of wood because the thermal conductivities of water vapor and air are very similar (Eitelberger and Hofstetter, 2011).

The factors above that significantly affect the thermal properties of wood were mostly determined by measuring the rate of heat flow at high temperatures (steady-state method); however, a literature review reveals limited information on the thermal conductive properties of wood at low temperatures. To expand the existing knowledge of the thermal conductive properties of wood at high temperatures, the present study investigates the relationship between the thermal conductive properties of wood at low temperatures and significant factors, namely, the microstructural

properties (density and porosity) and the anisotropy of wood using two types of solid carbon dioxide as a chilling agent. During this study the rate of heat flow could not be calculated; therefore, the thermal conductive properties of wood at low temperatures were defined as the cooling rate to the targeted critical thermal minimum (CT_{min}) of drywood termites ($^{\circ}C/min$), the minimum core temperature ($^{\circ}C$), and the duration of the temperature below CT_{min} (min).

5.2 Materials and methods

5.2.1 Wood Materials

Particular emphasis was placed on the selection of wood material. Accordingly, defect-free, normally grown wood materials were selected from four commonly used structural timbers in the Japanese wood market. As raw materials, four softwood species (moisture content ranged from 13 to 19%), Japanese cedar (*Cryptomeria japonica* D. Don), Japanese cypress (*Chamaecyparis obtusa* ENDL.), Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), and Sitka spruce (*Picea sitchensis* (Bong.) Carr.), air-seasoned for more than five years were used. The Japanese cypress and Sitka spruce specimens utilized in this study contained a considerable proportion of sapwood ($\geq 50\%$), whereas the other two species comprised 100% heartwood or a high percentage of heartwood ($> 75\%$), respectively (Table 5.1).

5.2.2 Preparation of the test blocks

Wood blocks of Douglas fir were cut from the large diameter of wood based on small ring curvature, whereas most of the other wood species were cut from a moderate diameter, which had a partially tangential and partially radial larger ring curvature. The wood blocks were then conditioned at $20 \pm 3^{\circ}C$ and $65 \pm 5\%$ relative humidity for eight weeks in a conditioning room until their weights stabilized. After the conditioning, the average values of the air-dried density

were calculated for each block based on the air-dried masses and volumes. To estimate the porosity (r) of the wood blocks, the following equation is given by Suleiman et al. (1999) was used:

$$r = 1 - (\rho_{ave}/\rho_{th}) \quad (1)$$

where ρ_{ave} = the average density of wood and ρ_{th} = the assumed theoretical density of a compact solid wood free from voids (1,500 kg/m³) (Kollmann and Côte Jr, 1968).

Afterwards, when the moisture content of the wood blocks reached below the fiber saturation point (< 30%), they were cut into dimensions of 85 (R) × 135 (T) × 200-mm (L) for tangential blocks and 85 (T) × 135 (R) × 200-mm (L) for radial blocks. To measure the instant temperature change of the wood block cores, a 3.5-mm diameter hole (70-mm deep) was drilled into the blocks from each cutting edge for thermocouple insertion (Fig. 5.1a). A total of 20 replications (five replicates/dry ice forms/cutting directions) were prepared from each wood species. The average microstructural and anisotropy data are presented in Table 5.1.

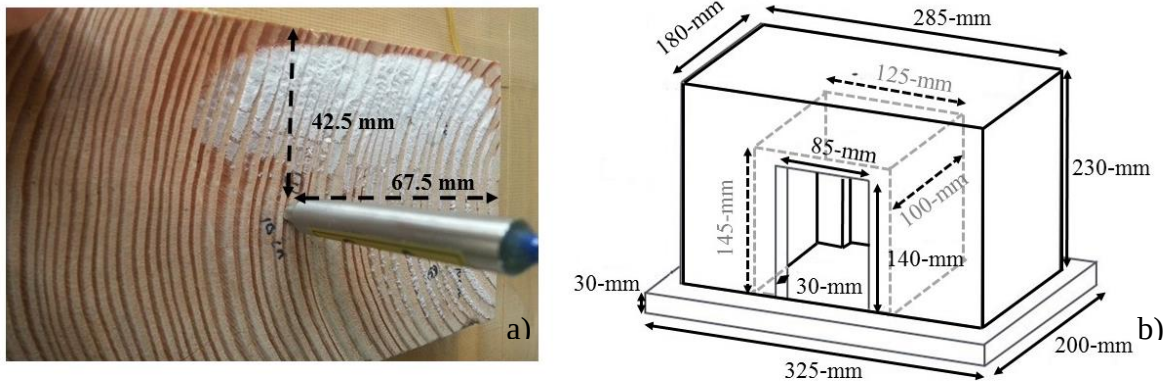


Figure 5.1 a) Instant temperature change of the wood block core on radial block b) Polystyrene insulation chamber

Table 5.1 Density, porosity, moisture content, growth ring and heartwood proportion measured and computed for the samples of the different blocks of wood in air- dry conditions (Mean $\pm$ SE)

Species	Direction	MC (%)	Density (kg m ⁻³)	Porosity (r)	Growth ring (#)	Ring angle (°)	Heartwood/ sapwood ratio (%)
Japanese cedar	Radial	21.7 $\pm$ 1.8	381 $\pm$ 9	0.746 $\pm$ 0.006	21.7 $\pm$ 1.8	22.4 $\pm$ 1.8	$\geq$ 75
	Tangential	18.8 $\pm$ 1.6	358 $\pm$ 8	0.761 $\pm$ 0.006	18.8 $\pm$ 1.6	23.0 $\pm$ 2.3	$\geq$ 75
Japanese cypress	Radial	14.8 $\pm$ 1.4	480 $\pm$ 15	0.680 $\pm$ 0.020	14.8 $\pm$ 1.4	61.8 $\pm$ 3.7	$\geq$ 50
	Tangential	16.3 $\pm$ 0.7	474 $\pm$ 7	0.684 $\pm$ 0.005	16.3 $\pm$ 0.7	61.0 $\pm$ 3.6	$\geq$ 50
Sitka spruce	Radial	16.8 $\pm$ 0.6	438 $\pm$ 9	0.708 $\pm$ 0.006	16.8 $\pm$ 0.6	62.2 $\pm$ 4.8	$\geq$ 50
	Tangential	17.9 $\pm$ 0.4	439 $\pm$ 4	0.707 $\pm$ 0.003	17.9 $\pm$ 0.4	59.0 $\pm$ 3.1	$\geq$ 50
Douglas fir	Radial	14.8 $\pm$ 1.4	564 $\pm$ 26	0.624 $\pm$ 0.017	14.8 $\pm$ 1.4	30.8 $\pm$ 1.5	100
	Tangential	16.3 $\pm$ 0.6	645 $\pm$ 9	0.570 $\pm$ 0.006	16.3 $\pm$ 0.6	29.0 $\pm$ 2.9	100

5.2.3 Measurement procedures

The low thermal conductive properties of the wood blocks were determined using two types of dry ice forms (solid slabs and crushed small particles/snow; $1,300 \pm 50$ g) in a specially designed polystyrene (JIS A-9511(JIS, 2009)) insulation chamber (Fig. 5.1b). The insulation chamber aimed to maximize the heat transfer properties of solid carbon dioxide. During the test operation, the instant temperature change of the wood blocks tested were monitored with a thermocouple (3.5 (Ø) × 70-mm (L), JUST Co. Ltd, Tokyo, Japan) connected to a computer via a thermocouple interface and SoftThermo E830 software (Technol Seven, Tokyo, Japan) for 20 hours at 15-min intervals.

5.2.4 Data analysis

To confirm the normality and homogeneity of the data variance, the Shapiro-Wilk test was used. Also, an analysis of variance (ANOVA) was used to test for significant differences between the two factors (wood species and grain angles) and levels. When the null hypothesis ($P < 0.05$) was rejected, a comparison of means was performed employing a Tukey HSD (Honestly Significant Difference) test at 5% of probability of error (SAS Institute, 2013).

5.3 Results

5.3.1 Influence of microstructure (density and porosity) on thermal conductive properties

The average cooling rates to the targeted critical thermal minimum (CT_{min}) of drywood termites ($^{\circ}C/min$), minimum core temperature ($^{\circ}C$), and duration of temperature below CT_{min} (min) of wood as a function of density and porosity are presented in Table 5.2. A positive correlation of thermal conductivity with density and porosity is shown to be partly coherent to the theory. For example, high energy is shown to be transferred through Douglas fir which had the highest density ($602 \pm$

46.8kg/m³) and the lowest porosity (0.599 ± 0.031), which is consistent with the relationship found by other studies. Meanwhile, Japanese cedar, which had the lowest density ($381.1 \pm 9.4 \text{ kg/m}^3$) and the highest porosity (0.753 ± 0.010) in the group, also exhibited higher energy transfer through the wood. Despite the density and estimated porosity according to Table 5.2 and the fact that the wood species must have different thermal properties regarding their thermal profiles, the cooling rate, minimum core temperature, and duration of those wood species showed no significant difference ($P < 0.0001$). Similarly, for the similar range of density and porosity of Japanese cypress ($431.4 \pm 11.9 \text{ kg/m}^3$; 0.712 ± 0.008) and Sitka spruce ($438.1 \pm 5.0 \text{ kg/m}^3$; 0.708 ± 0.003), the data showed significant differences in thermal properties at low temperatures. The mean cooling rate to target the critical thermal minimum (-21.3°C) for Sitka spruce was -0.23°C/min , whereas a cooling rate of only -0.19°C/min was recorded from Japanese cypress. Furthermore, the results of Japanese cypress for other associated parameters, such as minimum core temperature and duration of temperature below $CT_{\min}$, showed significantly higher and shorter results than Sitka spruce.

5.3.2 Influence of anisotropy on thermal conductive properties

According to the mean values of the thermal properties at the loading of 1,300 g of solid carbon dioxide in bar form for both the tangential and radial direction, the cooling rate increased in the radial direction in Douglas fir but did not significantly differ for the other thermal properties of wood at low temperatures (minimum core temperature and duration of temperature below $CT_{\min}$) compared with the tangential direction ($P < 0.0001$). The quickest cooling rates ($-0.27 \pm 0.1^\circ\text{C/min}$) were obtained in the radial direction of Douglas fir and both transverse directions of Japanese cedar followed by tangential Douglas fir and both transverse directions of Sitka spruce ($-0.23 \pm 0.1^\circ\text{C/min}$). The lowest values were obtained in both transverse directions in Japanese

cypress as $-0.19 \pm 0.0^\circ\text{C}/\text{min}$. For all wood species except Douglas fir, there was no significant difference in the cooling rate between the radial and tangential directions ($P < 0.0001$).

The increase in cooling rate and duration was similar to the order of the cooling rate. The lowest core temperature and the longest length of the $CT_{\min}$ temperature range were obtained from Douglas fir in the radial direction, and the highest core temperature and the shortest duration was obtained from both transverse directions of Japanese cypress (Fig. 5.2).

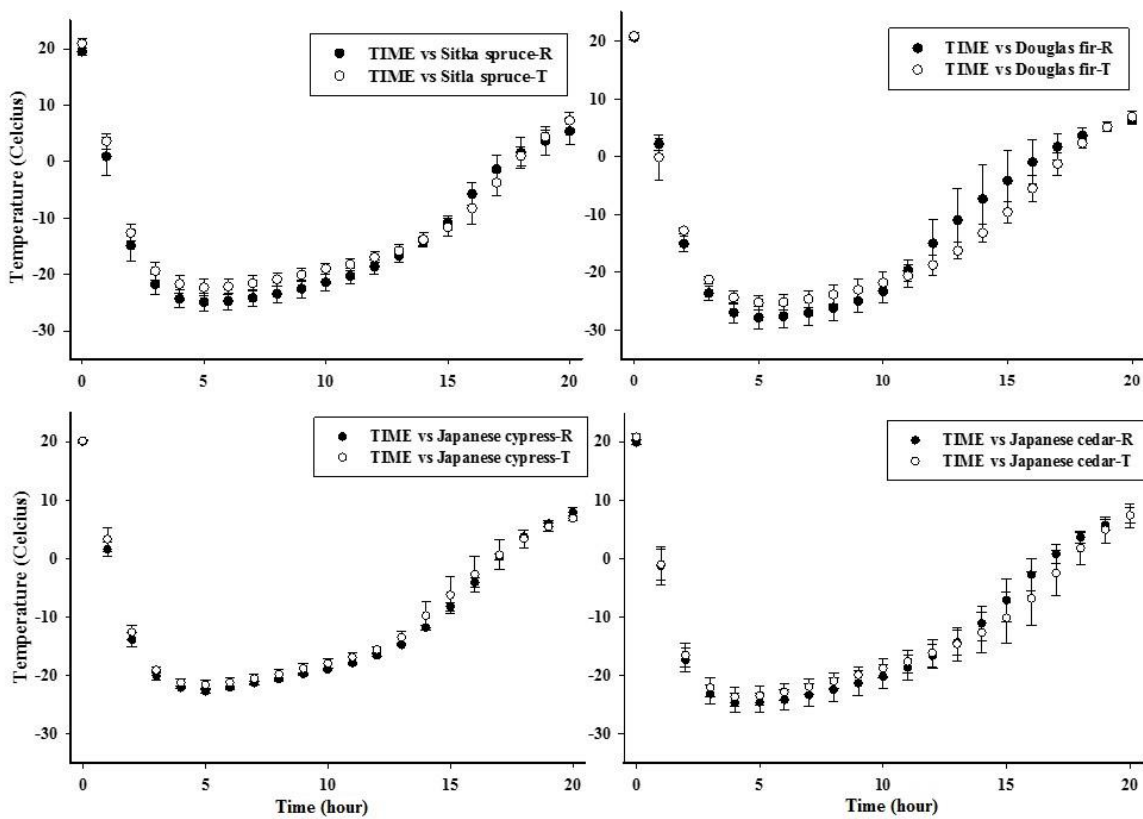


Figure 5.2 Comparison of the thermal conductive properties values obtained in both tangential and radial direction using solid carbon dioxide slabs

Table 5.2 Results of the thermal conductive property measurements performed with the solid carbon dioxide as chilling agent for commonly used structural timber species in transverse directions (Mean $\pm$ SE)

Species	Direction	Density (kg/m ³)	Porosity (r)	Cooling rate (°C/min)	Min. temperature (°)	Duration in CT _{min} temp. range (min)
Japanese cedar	Radial	381 $\pm$ 9	0.746 $\pm$ 0.006	-0.27 $\pm$ 0.00	-22.5 $\pm$ 0.7	490 $\pm$ 13
	Tangential	358 $\pm$ 8	0.761 $\pm$ 0.006	-0.26 $\pm$ 0.01	-25.2 $\pm$ 0.8	453 $\pm$ 22
Japanese cypress	Radial	480 $\pm$ 15	0.680 $\pm$ 0.020	-0.19 $\pm$ 0.00	-22.0 $\pm$ 0.6	180 $\pm$ 8
	Tangential	474 $\pm$ 7	0.684 $\pm$ 0.005	-0.19 $\pm$ 0.00	-21.6 $\pm$ 0.6	170 $\pm$ 6
Sitka spruce	Radial	438 $\pm$ 9	0.708 $\pm$ 0.006	-0.24 $\pm$ 0.01	-24.2 $\pm$ 0.3	420 $\pm$ 11
	Tangential	439 $\pm$ 4	0.707 $\pm$ 0.003	-0.23 $\pm$ 0.01	-23.3 $\pm$ 0.7	398 $\pm$ 8
Douglas fir	Radial	564 $\pm$ 26	0.624 $\pm$ 0.017	-0.27 $\pm$ 0.01	-26.4 $\pm$ 1.0	420 $\pm$ 11
	Tangential	645 $\pm$ 9	0.570 $\pm$ 0.006	-0.24 $\pm$ 0.01	-25.2 $\pm$ 0.9	490 $\pm$ 16

5.3.3 Influence of solid carbon dioxide forms on thermal conductive properties

Figure 5.3 compares the thermal conductive properties (cooling rate, minimum core temperature, and duration of temperature below CT_{min}) obtained in both the tangential and radial direction from two different solid carbon dioxide forms in this study. It appears that crushed small particles/snow solid carbon dioxide increases the thermal conductive properties of the wood species tested. The rate of convection heat transfer was obtained using Newton's law of cooling equation. The mean values of 0.146W/m^2 were generated by crushed solid carbon dioxide, whereas 0.138W/m^2 was produced by solid carbon dioxide slabs. This phenomenon might be caused by the faster sublimation of small particle/snow which extracts the heat energy from the immediate surroundings resulting in extreme cooling compared with solid carbon dioxide slabs. Consequently, the crushed form of solid carbon dioxide increased the cooling rate by a minimum of 9.9% to a maximum of 28.9%, the minimum core temperature by a minimum of 7.7% to a maximum of 10.6%, and the duration by a minimum of 8.8% to a maximum of 17.0% compared with solid slabs.

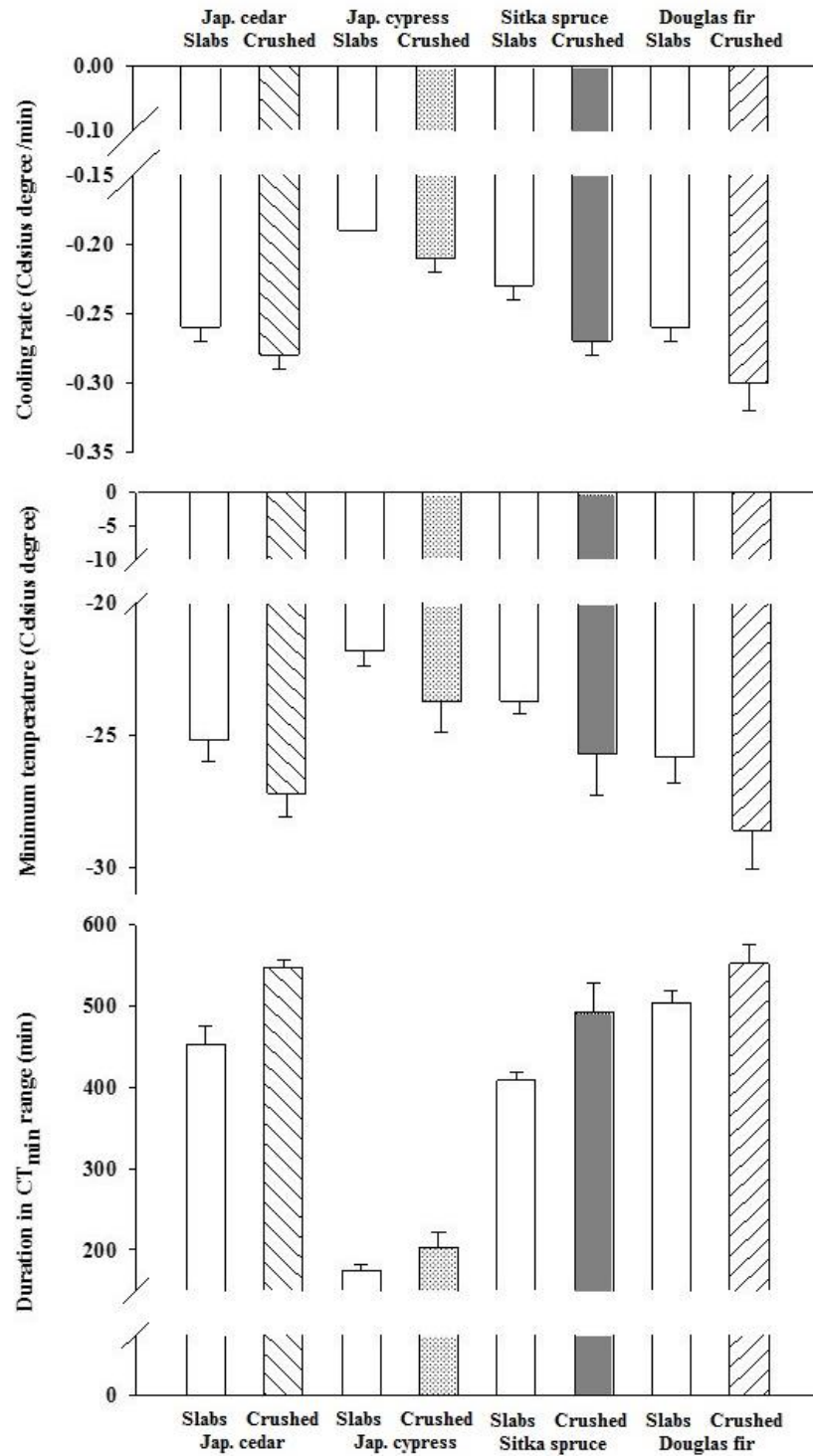


Figure 5.3 Comparison of the thermal conductive properties values obtained in two type of solid carbon dioxide form

5.4 Discussion

Based on the theory of the thermal conductive properties of wood, higher densities, and lower porosities increase the thermal conductive properties of wood (Suleiman et al., 1999; Yu et al., 2011). However, in contrast to the thermal conductive properties of wood at high temperature, few discrepancies were observed at low temperatures.

The results indicate that the thermal conductive properties of wood at low temperature are mainly affected by the composition and morphological properties of wood (i.e., density/ratio of earlywood and latewood, the proportion of heartwood and sapwood, the pattern of growth ring). In Figure 1a, the cross-section photo reveals a clear frost variation where high energy is shown being transferred first through the latewood ring and then across into the earlywood ring. In the latewood of softwood, thick cell walls with a small lumen increase the thermal conductive properties because of heat transfers mainly through cell walls (Gu and Zink-Sharp, 2005). By contrast, earlywood has thin walls with larger cell lumens. Below the FSP, these cell lumens are mostly filled with air, which consequently decreases the thermal properties of wood due to the low thermal properties of air (Asako et al., 2002). A study of the thermal conductive properties below the FSP by Gu et al. (Gu and Zink-Sharp, 2005) also revealed that the percentage of latewood substantially affects the thermal conductive properties of wood. For example, for spruce, the densities of early wood and late wood differ by three factors (Sonderegger et al., 2011).

Thermal conductive properties may also be responsible for the differences between heartwood and sapwood. The higher proportions of heartwood of Douglas fir (100%) and Japanese cedar (>75%) recorded higher thermal conductive properties, which may indicate that the chemical substances in heartwood increase the thermal conductive properties. MacLean and others (Lacoste

et al., 2013; MacLean, 1941; Suleiman et al., 1999) deduced that chemical substances in the heartwood such as tannin, resin or oil possibly increase the thermal conductive properties.

The effect of anisotropy on the thermal conductive properties of wood is a highly controversial topic. Some scientists claim that the thermal conductive values in the radial direction are higher than in the tangential direction (Kollmann and Côte Jr, 1968; Niemz et al., 2010; Sonderegger et al., 2011), whereas others report the same thermal conductive properties in both transverse directions (Simpson and TenWolde, 1999; Steinhagen, 1977; Suleiman et al., 1999). However, most importantly, the thermal conductive properties are mainly dependent on the pattern of the growth ring itself at low temperatures. In the case of Douglas fir, the thermal conductive properties in the radial direction are higher than in the tangential direction. However, in contrast with Douglas fir, similar thermal conductive properties in the tangential and radial directions were obtained for moderate-diameter wood, which comprises partly tangential and partly radial directions. Consequently, this combination of both transverse directions influences the anisotropic factor of the thermal conductive properties in this study. Furthermore, the orientation of the microfibrils strongly affects the thermal conductive properties. Some other studies have reported that the thermal conductive properties of wood increase with increasing ring angle which is influenced by the microfibril angle in the S2 layers of the cell walls (Niemz et al., 2010; Sonderegger et al., 2011; Vay et al., 2013).

The results have shown that the thermal conductive properties of wood at low temperatures are mainly dependent on the hierarchical organization of the wood. Therefore, to establish a model that can predict the thermal conductive properties at low temperatures, the thermal conductive properties of the cell wall material are required in a future study. Also, the effect of knots, checks, and other defects on structural timber must be explored because these defects have a tendency to

increase the thermal conductive properties of wood. Finally, in addition to the three parameters above, the probe location strongly influenced the results due to the localized temperature measurement and the impact of heterogeneities. Hence, the probe location must be carefully selected to eliminate the influence of heterogeneities and also to increase the number of probe locations to encompass the temperature measurement of the entire sample in the future study. Overall, as remedial drywood termite treatments using solid carbon dioxide are developed to kill them inside timber, we are confident that the results obtained demonstrate the high capability of the application.

CHAPTER 6: Novel application of a two-in-one bandage system for improving remedial and preventive treatment of drywood termite control

This chapter has been submitted to Pest Management Science.

6.1 Summary

The development of verifiable detection technology allows us to pinpoint an infested area and confirm the drywood termite control application efficacy. With the development of this detection technique, the greatest area of improvement of insecticide has been touted. In contrast, the technology associated with drywood termite management has remained unchanged for the past decades.

We assume that the combination of two or more treatment approaches with different mechanisms can cooperatively prohibit drywood termite eradication and prevent re-infestation. Our two-in-one bandage system provides a composition comprising a remedial treatment using carbon dioxide snow and a preventive treatment using imidacloprid. Our assessment of the effectiveness of the remedial treatment was highly influenced by the structure of the wood and the dosage of the carbon dioxide snow. The surface deposit layer and the diffuse through the wood created after the preventive treatment in the two-in-one bandage system were effective in the prevention of timber from re-infestation.

6.2 Introduction

The understanding of the foraging behavior of drywood termites improves early detection and monitoring and suggests more efficient control methods and follow-up monitoring. Recently, the greatest area of development has been devoted to studying the cryptic habits of drywood termites using computer tomography (CT) to determine their foraging pattern (Himmi et al., 2014; Perna

et al., 2008) and social behavior (Fuchs et al., 2004; Korb and Foster, 2010; Viana et al., 2013). The CT observations of forage patterns of drywood termites show that foragers primarily excavate along the Springwood towards the outer surface of the timber to establish the primary (ca. > 10-mm beneath the surface) and satellite chambers, followed by a mainly superficial (3 – 30 mm under the surface) longitudinal expansion and an occasional lateral direction expansion (Himmi et al., 2016). This verifiable detection technology allows us to pinpoint an infested area and confirm the control application efficacy. With the development of this detection technique, the greatest area of improvement of insecticide has been touted with interest in the environment and human health. The new insecticides appear to have great promise in pest management due to a high degree of selectivity, low toxicity to non-target organisms and high safety for the environment. In recent years, the most promising areas of insecticide development have been the identification of compounds acting on insects' nicotinic acetylcholine receptors (nAChRs) (Matsuda et al., 2001). Imidacloprid is one of the neonicotinoid insecticides that shows excellent systemic properties and is effective against agriculture and urban pests (Elbert et al., 1991).

In contrast, attention to the development of the novel application has not been devoted. The technology associated with drywood termite management has remained unchanged for several decades. Localized agrochemical treatments, either by injection or surface treatment, dominates the market share with 70% for drywood termite control (Potter, 2004) which highly relies on the efficacy of accurate detection of galleries and chambers and has strong insecticidal properties. The future trends of controlling the drywood termite will rely more on local treatment due to the improvement in detection technology, a high degree of selectivity, low toxicity to non-target organisms, and safety for the environment. Non-chemical drywood termite control applications have developed some concerning ectothermic characteristics of the drywood termite. Warm-

blooded insects' body temperature reflects the temperature of the surrounding environment (Harrison et al., 2012). Based on the idea of natural law, many researchers have conducted studies regarding the management of drywood termites (Forbes and Ebeling, 1986; Lewis and Haverty, 1996; Rust et al., 1997) and other pests (Donahaye, 2000; Florian, 1990; Gilberg and Brokerhof, 1991; Kells, 2006). For the last eight decades, heat treatment has been the most representative non-chemical treatment for controlling the structural pests. At the opposite end of the temperature spectrum, cryogen (i.e., liquid nitrogen and carbon dioxide) has been tested in both laboratories and fields against the drywood termite since the 1980s. The outcome of these studies has allowed the pest management company to develop the remedial treatment for drywood termite (Blizzard[®]) and other urban pests (Cryonite[®]).

Drywood termite control applications relying on a single treatment remain a suboptimal method since complete protection is not possible in thermal applications because there is no residual protection. We assume that the approach of a combination of two or more treatments with different mechanisms can cooperatively prohibit drywood termite eradication and prevent re-infestation. The purpose of this study was to develop a two-in-one drywood termite control application that will evolve into a remedial treatment using carbon dioxide snow and preventive treatment, Imidacloprid.

6.3 Materials and methods

6.3.1 Influence of structure on thermal conductive properties of wood at low temperatures

Air-dried structure timbers with a moisture content below 22% were selected for this study. Three replications of timber blocks, Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Japanese cypress (Hinoki: *Chamaecyparis obtusa*), were sawed out from near the border of the heartwood and

sapwood with the dimension of 85 (H) x 135 (W) x 1000-mm (L). Details of microstructural and anisotropy data are presented in Table 6.1.

The low thermal conductive properties of the wood blocks were determined using carbon dioxide snow (800, 1,000, and 1,200 g) in a specially designed polyurethane bandage system (Fig. 6.1). The polyurethane bandage system aimed to maximize the energy transfer properties of the carbon dioxide snow. The instant temperature change of the wood block was monitored with a 3.5-mm thermocouple (100-mm long; JUST Co. Ltd, Tokyo, Japan), which was inserted into the blocks from each cutting edge, connected to a computer via the interface of a thermocouple and SoftThermo E830 software (Technol Seven, Tokyo, Japan) for 12 hat 15-min intervals. In total, there were ten replications (five replicates per wood species).

Table 6.1 Results of the thermal conductive property measurements performed with carbon dioxide snow as a chilling agent for commonly used structural timber species (Mean $\pm$ SE)

Parameters	Japanese cypress			Sitka spruce		
Density (kg m ⁻³)	474.9 $\pm$ 6.8 ^a			436.9 $\pm$ 3.5 ^b		
Porosity (<i>r</i>)	0.683 $\pm$ 0.005 ^b			0.709 $\pm$ 0.002 ^a		
MC (%)	19.4 $\pm$ 0.3 ^a			20.9 $\pm$ 0.5 ^a		
Growth ring angle ¹ (°C)	48 $\pm$ 3 ^b			87 $\pm$ 2 ^a		
Growth ring (#)	15 $\pm$ 0 ^b			18 $\pm$ 1 ^a		
CO ₂ snow loading ⁴ (g)	800 (69.8 kg m ⁻³)	1,000 (87.2 kg m ⁻³)	1,200 (104.6 kg m ⁻³)	800 (69.8 kg m ⁻³)	1,000 (87.2 kg m ⁻³)	1,200 (104.6 kg m ⁻³)
Cooling rate ² (°C/min)	-0.13 $\pm$ 0.00 ^{b(a)}	-0.14 $\pm$ 0.00 ^{ab(a)}	-0.15 $\pm$ 0.01 ^{a(b)}	-0.13 $\pm$ 0.0 ^{c(a)}	-0.15 $\pm$ 0.01 ^{b(a)}	-0.17 $\pm$ 0.0 ^{a(a)}
Min. temperature ³ (°C)	-16.0 $\pm$ 0.7 ^{c(a)}	-19.8 $\pm$ 0.6 ^{b(a)}	-23.0 $\pm$ 0.7 ^{a(b)}	-15.9 $\pm$ 0.4 ^{c(a)}	-21.1 $\pm$ 1.0 ^{b(a)}	-25.5 $\pm$ 0.5 ^{a(a)}

Significant difference between wood species indicated by different letters (P < 0.05; paired t-test)

¹The growth ring angle was measured from the CO₂ snow depositing surface (radial section) with a protractor

^{2,3} Significant difference of three CO₂ snow loading within same wood species indicated by different letter (P < 0.05; Tukey's HSD)

⁴ Significant difference of CO₂ snow loading between wood species indicated by different letters in parentheses (P < 0.05; paired t-test)

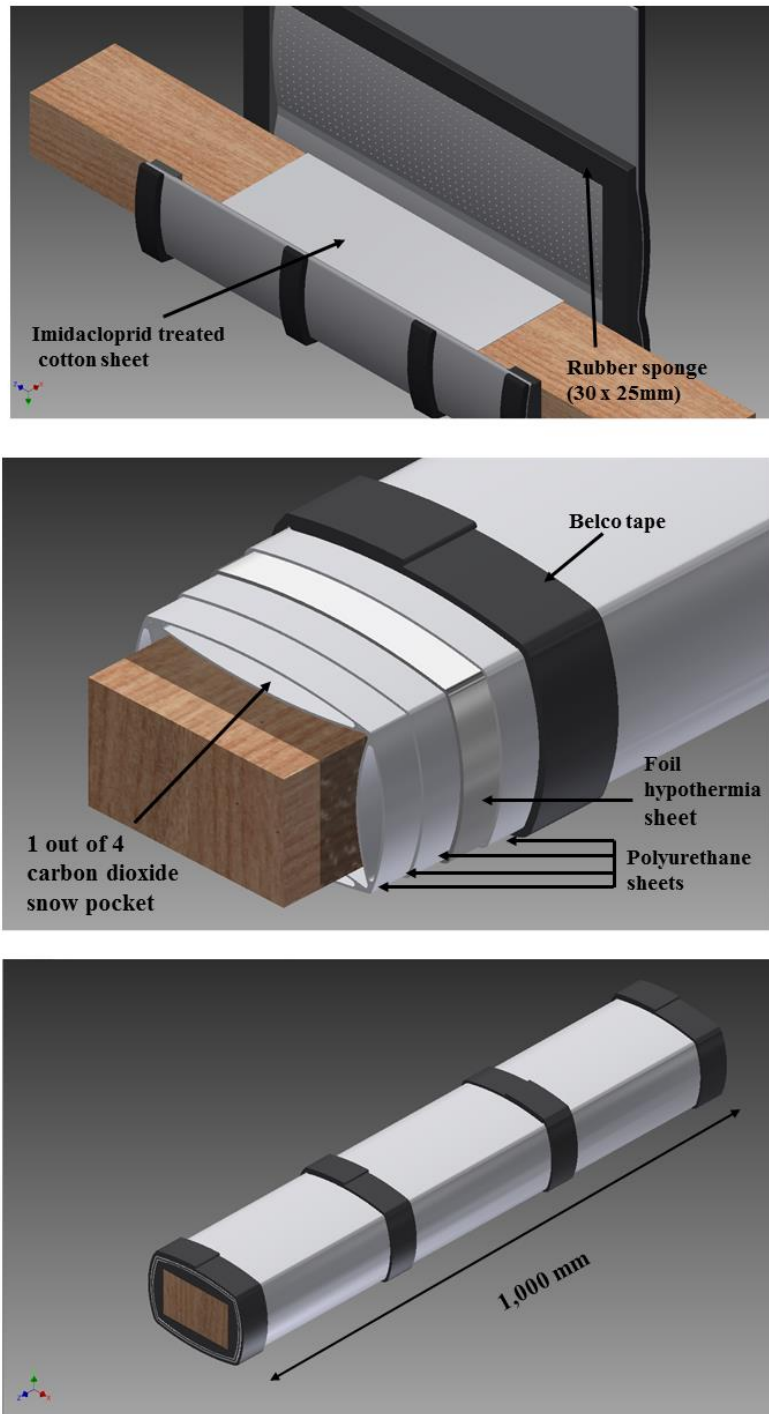


Figure 6.1 Test apparatus used for the evaluation of the remedial and preventive treatment for drywood termites

6.3.2 Determination of insecticidal activity of treated surfaces

Wood blocks, ca. 100 x 75 x 15 mm, were prepared from the same wood source as the thermal conductive properties test. Before chemical application, the wood blocks were conditioned in a conditioning room ($20 \pm 3^{\circ}\text{C}$ and $65 \pm 5\%$ RH) until their weights stabilized. After conditioning, the mean density was calculated based on air-dried weight and volume. The porosity (r) of the wood blocks were estimated based on Suleiman's equation (Suleiman et al., 1999):

$$r = 1 - (\rho_{\text{ave}}/\rho_{\text{th}}) \quad (1)$$

where ρ_{ave} = the average density of wood and ρ_{th} = the assumed theoretical density of a compact solid wood free from voids ($1,500 \text{ kg/m}^3$) (Kollmann and Côte Jr, 1968).

Three concentrations of imidacloprid, 0.05, 0.10, and 0.20%, were used to determine insecticidal activities. Imidacloprid of the highest purity (>97%), which was supplied by Bayer (Tokyo, Japan), was diluted in 160 ml acetonitrile (Nacalai Tesque, analytical grade) and 40 ml of distilled water (v/v) to prepare the four concentrated solutions. After the preparation of the fresh dipping solutions weighed cotton pads (ca. 3.0 g; 100 x 75 mm) with a thickness of 1–2 mm were dipped into the solutions and immediately weighed for uptake calculations and then placed on the surface of the wood block. After the application of the treated cotton pad, 30 g of carbon dioxide snow was placed on top of the cotton pad and the whole specimen was wrapped with wrap film. After 12 h of the envelope treatment with carbon dioxide snow, the cotton pads were weighed to estimate the remaining active ingredient in the cotton pads. Meanwhile, enveloped treated wood blocks were air-dried for one week in a dark chamber before placing ten healthy workers (10 mg body mass or heavier) on the surface of the enveloped treated wood block (Fig. 6.2). In total, 30 replications

(five replicates per dosage per wood species) were prepared for continuous exposure to determine the insecticidal activities of treated timber.

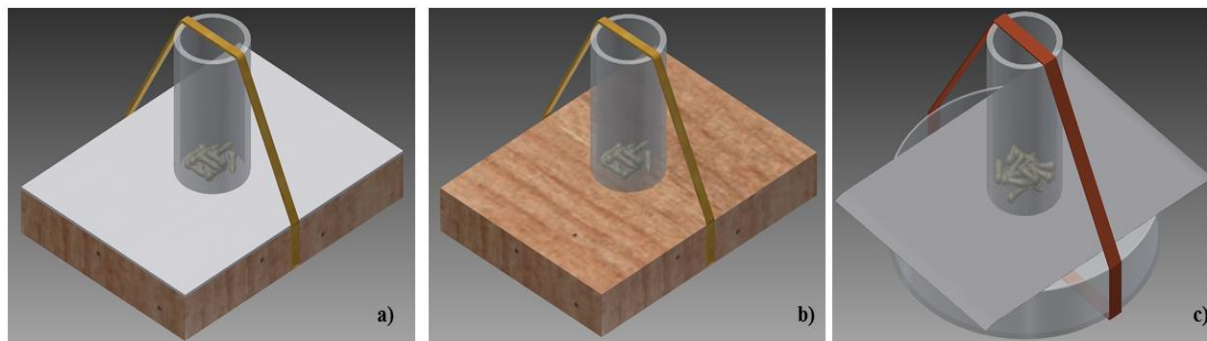


Figure 6.2 Experimental setup for the insecticidal activity of a treated wood block. a) untreated cotton pad after envelope treatment with CO₂ snow, b) treated wood block, and c) imidacloprid treated cotton pad after envelope treatment with CO₂ snow

To evaluate the efficacy of the preventive treatment, individuals were exposed for seven days with daily evaluation. Mortality was rated according to an arbitrary 0–3 scale as follows: 0 = normal, 1 = intoxicated, 2 = moribund, and 3 = dead. Normal termites showed free activity and were able to flip their body back onto their feet within 1 min; intoxicated individuals showed hyperactivity or paralysis of body and took more than 1 min to flip their body back onto their feet; moribund termites exhibited only slight twitching and were unable to right themselves, whereas dead termites showed no response to touch.

6.3.3 Analysis of remaining active ingredient in the cotton pad (0.2%) samples

Diffusion of the active ingredient was analyzed with six treated cotton pads (three replicates per wood species) after a toxic threshold assessment and six treated cotton pads without the application of carbon dioxide snow (as control). Scraps of the cotton pad were placed into 100 ml a measuring flask to which 80 ml acetonitrile and 20 ml distilled water (v/v) solution was added. The measuring flask was left in ultrasonication for 30 min to allow imidacloprid to extract. A repeat extraction

procedure was performed with 80 ml acetonitrile and distilled water and applied to HPLC (HP 1100, Agilent Technology, Palo Alto, CA, USA) equipped with a narrow-bore column (Mighysil RP-18; 2 mm x 12 cm, 5 μ m; Kanto Chemical, Tokyo, Japan). The moving phase was composed of acetonitrile and distilled water (17.5/82.5; v/v), at a flow rate of 0.20 ml/min. The column temperature was held at 30°C. The injection volume was 1 μ l, and the UV absorbance was measured at 270 nm. Data acquisition and analysis was achieved using HP ChemStation software (Agilent Technology, Palo Alto, CA, USA).

6.3.4 Two-in-one bandage system effects on mortality of drywood termites

Artificial infestation of structure timber blocks was placed against active *Incisitermes minor* (Hagen) workers, who were collected from naturally infested wooden structures in Wakayama Prefecture, Japan. Each wood species replication, 70 healthy workers (10 mg body mass or heavier) was carefully selected after one week of the stabilization period ($28 \pm 2^\circ\text{C}$, >85% RH). After the introduction of workers, the blocks were kept in the dark drywood termite sample room ($28 \pm 2^\circ\text{C}$, and > 85% RH) for 12 months. During the first two weeks of the exploratory activity, the mortality of the workers in the releasing chamber was recorded daily.

Twelve-month-old infested timber was used to evaluate the remedial and preventive treatment for drywood termites. As a preventive treatment, 0.2% Imidacloprid cotton pads were prepared in the same way as the insecticidal activity assessment procedure. Cotton pads consisting of 0.2% Imidacloprid were first placed on the surface of infested and suspected infested areas of timber. After the preventive treatment, the remedial treatment (bandage application system composed of four polyurethane sheets and a foil hypothermia sheet; Fig 6.1) was wrapped around the timber, followed by a carbon dioxide snow injection using the Cryonite[®] rifle system. A total of 1,200 g of carbon dioxide snow was injected into the bandage application system. After 12 h of the two-

in-one bandage system, the bandage application system was unfasted, and the timbers and cotton pads were air-dried separately for a week. The indices of mortality of the 12 h remedial treatment were determined by separating the dead and live workers.

To assess the results of the efficacy of the preventive treatment, three sets of ten healthy workers (10 mg body mass or heavier) were placed on the surface of the sawn timbers of the artificially infested timber (Fig 6.3). In total, 18 replications (three replicates per number of artificially infested timber per wood species) were used, and three replications of untreated cotton pads were used as the control.

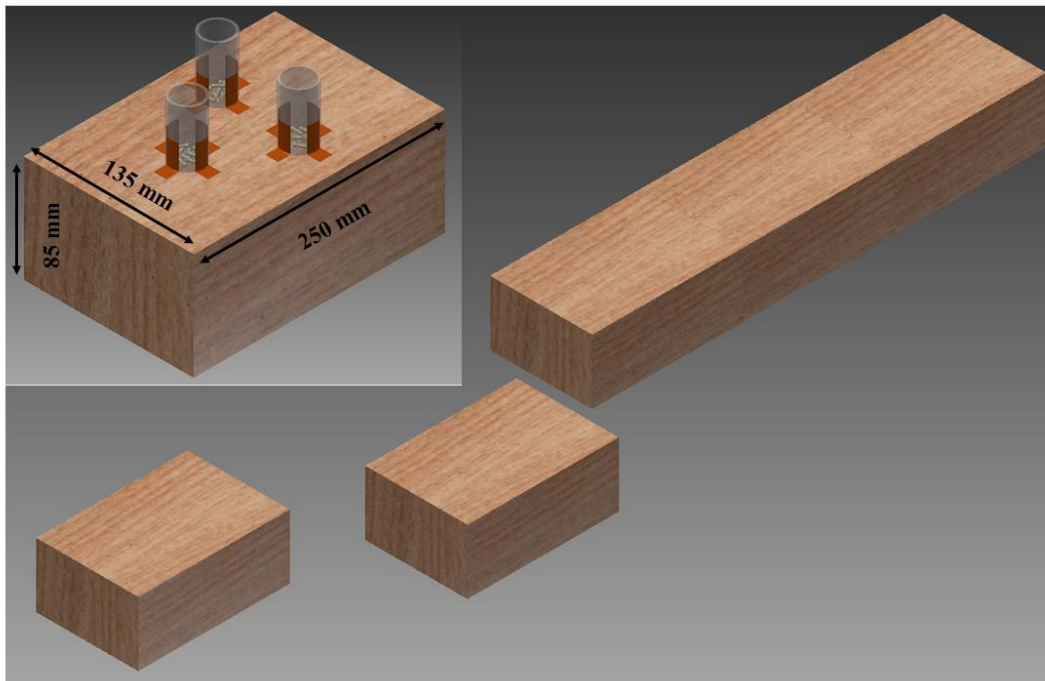


Figure 6.3 Experimental setup for the preventive treatment using one year artificially infested timber after remedial treatment

6.3.5 Data analysis

All percentage data were arcsine square root transformed before an analysis of variance (ANOVA) was performed. To determine the difference between loading amounts of carbon

dioxide snow (thermal conductivity assessment) and concentrations of active ingredients (insecticidal activity assessment), an ANOVA was performed, and the mean values were separated using Tukey's honestly significant difference (HSD) test. The possible effects of carbon dioxide snow on the diffusion of imidacloprid were explored using a paired t-test. We examined the efficacy changes over time to evaluate how quickly a given dosage of imidacloprid suppressed drywood termite workers. Probit analyses (Pearson goodness of fit Test) were conducted to correlate the change in mortality and exposure period. Finally, the differences in the remedial treatment mortality levels and preventive treatment mortality levels in Sitka spruce and Japanese cypress were also explored using a paired t-test. All statistical analyses were conducted using SPSS version 18 (SPSS, 2009).

6.4 Results

6.4.1 Influence of structure on thermal conductive properties of wood at low temperature

The average cooling rates ($^{\circ}\text{C}/\text{min}$) and minimum core temperature ($^{\circ}\text{C}$) of wood as a function of microstructures are presented in Table 6.1. Paired t-tests indicated a positive correlation of thermal conductivity with density, and porosity was not coherent to the theory, whereas, growth ring angle ($t = 10.285$, $P = 0.0005$) and growth ring number ($t = 4.000$, $P = 0.0161$) showed a positive correlation of thermal conductivity of wood. Until there was 1,000 g of CO_2 snow loading, no significant difference ($P > 0.0001$) on thermal conductive properties of wood were observed corresponding to the growth rings. The influence of the growth rings on the thermal conductivity began at 1,200 g of CO_2 snow loading. Sitka spruce samples with significantly steeper growth ring angles ($>85^{\circ}$) and a higher consistency of growth rings (18 ± 1), showed that higher energy was transferred into the core of timber than Japanese cypress (Fig. 6.5). In consequence of ca. 11.8%

quicker cooling rates and ca. 9.8% lower minimum core temperature were observed. When we injected CO₂ into a bandage application system, we obtained $35.6 \pm 0.8\%$ deposition of CO₂ snow.

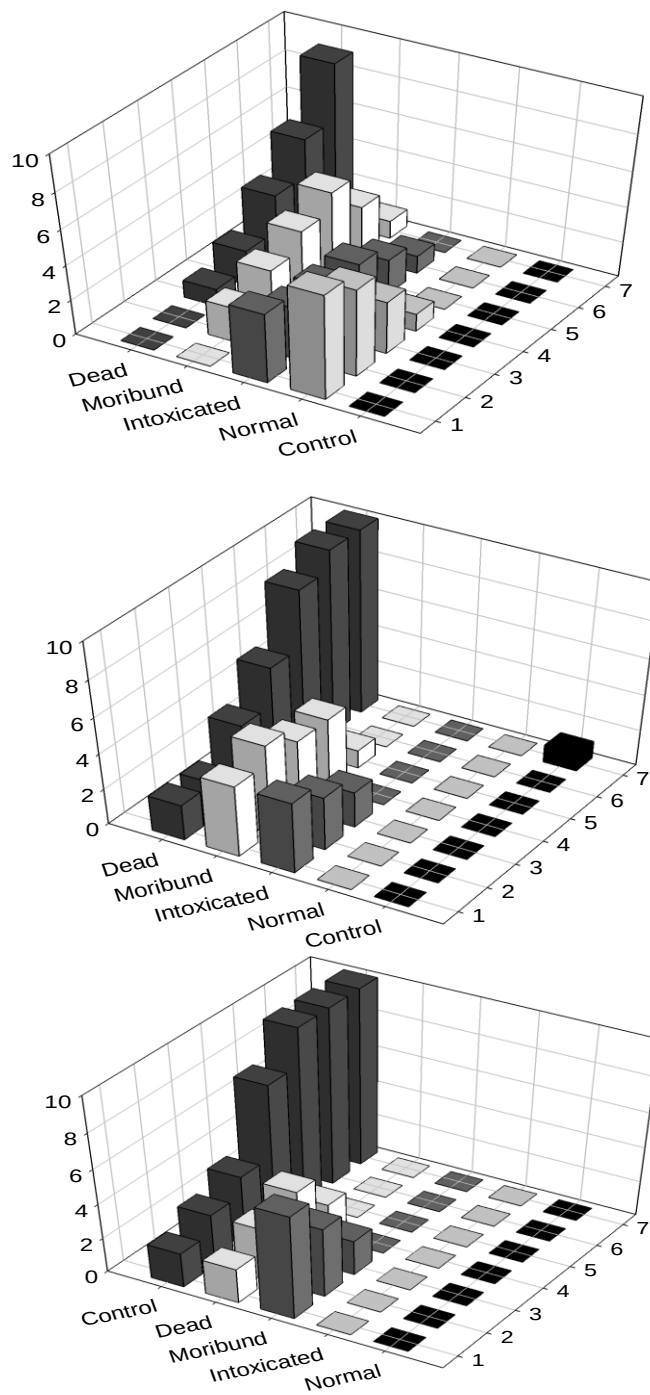


Figure 6.4 Number of termite workers, out of 10, showing progressive poisoning symptoms during the 7 days of access to treated wood blocks consisting of a) 0.05%, b) 0.1%, and c) 0.2% imidacloprid

6.4.2 Determination of insecticidal activity of treated surfaces

Individual drywood termites were observed on imidacloprid-treated wood blocks. The concentration-response relationship in drywood termites was affected by the increasing dosage of imidacloprid (Fig 6.4). When drywood termites were exposed to 0.2% imidacloprid, individuals succumbed to the toxic's effects between day 3 (40% moribund and 40% dead) and day 5 (100% dead). The survival probability for the drywood termite workers at lower a dosage improves. At the lowest dosage (0.05%) tested, more than half of individuals were considered as survival (30% normal and 30% intoxicated) after day 3. In a comparison of the toxic's effects after day 3, there was a significant difference among concentrations of imidacloprid ($F_{2,27} = 87.828$, $P < 0.0001$). In lower dosages, all drywood termite cohorts died after day 5 (0.1% a.i.) and day 8 (0.05% a.i.).

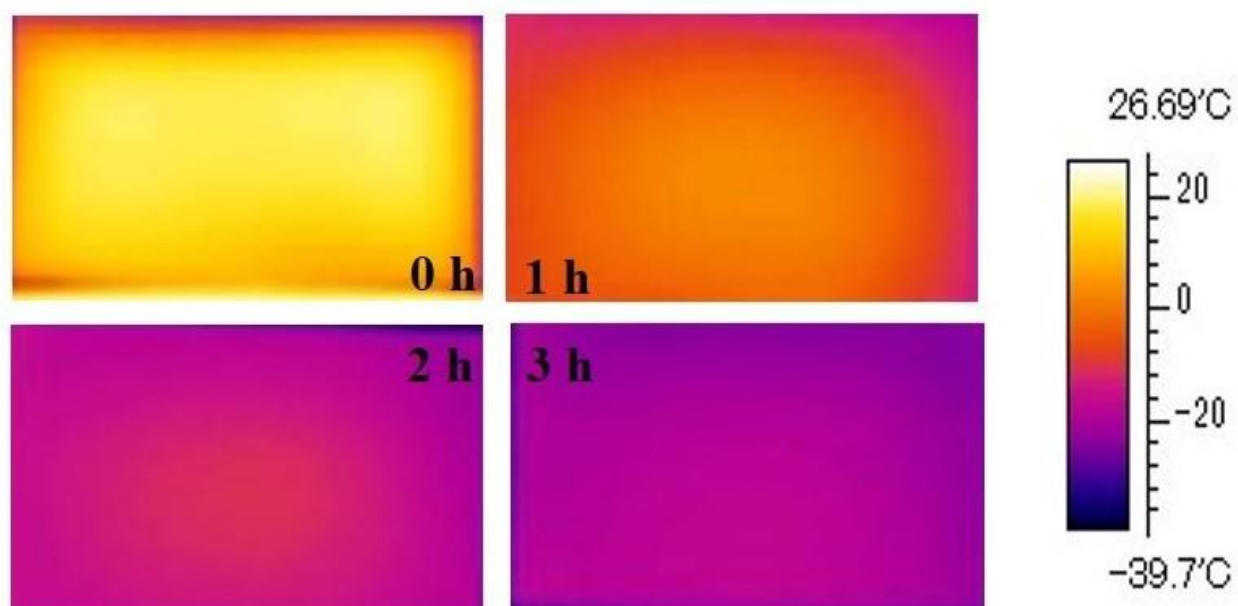


Figure 6.5 Temperature pattern changes in Sitka spruce timber over a 3 h period in a two-in-one bandage system

We observed behavioral symptoms associated with imidacloprid, such as signs of disorientation, increased walking speed, horizontal oscillatory movement, and bent beneath the mouthparts in an early stage at a low dosage. During the later stage, workers exposed to a low dosage of

imidacloprid often circled the same spot, appeared collapsed or released liquid excrement (yellow spots) and proctodeal fluid (dark spots). Workers immediately showed an alarmed reaction as a behavioral symptom associated with a higher dosage. Intoxicated workers walked in reverse, exhibited whole body movements accompanied with leg kicking, weak movements of the antennae and eventually sluggish movements. Workers then remained on their feet or back and released liquid excrement and proctodeal fluid.

6.4.3 Analysis of remaining active ingredient in the cotton pad (0.2% a.i.) samples

The initially estimated uptake of imidacloprid in the cotton pad was 11.7 ± 0.2 mg of imidacloprid. After the envelope treatment with and without CO₂ snow, a higher percentage of imidacloprid on the cotton pad was allowed to migrate to the wood. Based on HPLC analysis, the estimated remaining imidacloprid in the cotton pad used on Sitka spruce and Japanese cypress were 0.44 ± 0.04 mg (ca. 3.7%) and 0.38 ± 0.02 mg (ca. 3.4%), respectively, and showed no significant difference ($t = 1.228$, $P = 0.2869$). Similar to the envelope treatment with CO₂ snow, there were higher percentages (> 95%) of removal obtained in the cotton pads from envelope treatments without CO₂ snow. Paired t-tests showed that there was no significant difference between wood species and enveloped CO₂ snow treatments, which removed imidacloprid from the cotton pad to the surface of wood ($P > 0.05$). Table 6.2 shows remaining a.i. of imidacloprid in the treated cotton pad after an envelope treatment with 30 g of CO₂ snow.

Table 6.2 HPLC analysis results of the remaining imidacloprid (0.2% a.i.) in cotton pads performed with and without 30 g of carbon dioxide snow

Species	Status*	Cotton pad (mg)	Uptake quantity (mg)	Residual quantity in cotton pad (mg)	Residual quantity in cotton pad (%)	Statistic*
Sitka spruce A	With CO ₂ snow	296.3	11.51	0.53	4.6%	$t = 1.0314,$ $P = 0.3606$
Sitka spruce B	With CO ₂ snow	276.8	9.77	0.39	4.0%	
Sitka spruce C	With CO ₂ snow	300.3	11.02	0.41	3.7%	
Japanese cypress A	With CO ₂ snow	297.8	10.38	0.4	3.9%	
Japanese cypress B	With CO ₂ snow	284.1	9.91	0.34	3.4%	
Japanese cypress C	With CO ₂ snow	281.2	10.74	0.41	4.0%	$t = -1.5811,$ $P = 0.1890$
Sitka spruce A	Without CO ₂ snow	300.4	11.22	0.39	3.5%	
Sitka spruce B	Without CO ₂ snow	290.5	10.60	0.39	3.7%	
Sitka spruce C	Without CO ₂ snow	297.6	10.56	0.38	3.6%	
Japanese cypress A	Without CO ₂ snow	330.5	11.17	0.44	3.9%	
Japanese cypress B	Without CO ₂ snow	281.9	10.30	0.39	3.8%	
Japanese cypress C	Without CO ₂ snow	284.5	10.77	0.39	3.6%	

* Residual quality (%) of each status between wood species was statistically compared using paired t-test ($P < 0.05$)

6.4.4 Two-in-one bandage system effects on mortality of drywood termites

Remedial treatment using 1,200 g of CO₂ snow generated a lethal temperature to control drywood termites in 0.0115 m⁻³ of both structure timbers. Based on an infrared camera image (Fluke-TiS40, Everett, WA) taken in first 3 hours, the core temperature of timber reached the lethal temperature (−21.3°C) for drywood termites (Fig 6.5). After the 20 h cryogenic treatment, we determined that 100% mortality was achieved from the remedial treatment. Within 100% mortality of drywood termites, more than 60% of the dead workers were recovered near the primary and satellite chambers where workers primarily excavate along the springwood (radial pattern) towards the outer surface of the wood, and rest of them were recovered from extensive galleries beneath the surface of the wood. The assessment of the effectiveness of the remedial treatment in a two-in-one bandage system with 1,200 g of CO₂ snow was highly efficient and resulted in eradication. The difference in mortality between Sitka spruce and Japanese cypress were not significant (pair t test, $P > 0.05$) after the 20 h remedial treatment (Table 6.3a).

The effects of the preventive treatment experiment have demonstrated that the deposited imidacloprid after the remedial treatment can be lethal to drywood termites. All artificially infested structural timbers in the preventive treatment with 0.2% imidacloprid can effectively prevent a drywood termite attack. The initial imidacloprid efficacy was classified as moderate (ca. 45.5%) on Sitka spruce and high (ca. 92.2%) on Japanese cypress. Table 3b shows that mortality levels (dead and moribund) at day 1 in Sitka spruce timbers were significantly lower than Japanese cypress ($t = -4.529$, $P = 0.0106$). However, the differential mortality level gap tends to diminish as exposure time increases. Three days after exposure, the mortality levels in Sitka spruce and Japanese cypress were recorded at 86.7% and 96.7%, respectively ($t = -2.774$, $P = 0.0502$). After 4 days exposure, mortality levels exceeded the 100% level of efficacy in all artificially infested

structure timbers. The cumulative mortality after seven days using by probit analysis shows that the lethal time to death (LT_{50} and LT_{95}) with 95% confidence intervals of workers on Sitka spruce and Japanese cypress were 24.2 h and 116.5 h (slope $\pm$ SE: 2.56 ± 0.43 ; $P < 0.0001$), and 1.2 h and 41.9 h (slope $\pm$ SE: 1.06 ± 0.66 ; $P = 0.106$), respectively.

Table 6.3 Overall percentage of the mortality of workers after a two-in-one bandage system in artificially infested structural timbers a) remedial treatment b) preventive treatment

a) Remedial treatment

Species	Mortality (mean $\pm$ SE)	Location of dead body recovery		
		Within 25 cm Primary chamber	25~ 50 cm Primary chamber	>50 cm Primary chamber
S. spruce	100 $\pm$ 0	73.5 $\pm$ 3.4	21.9 $\pm$ 1.9	5.9 $\pm$ 5.9
J. cypress	100 $\pm$ 0	87.3 $\pm$ 7.1	12.7 $\pm$ 7.1	0

b) Preventive treatment

Species	Day 1				Day 2				Day 3				Day 4				Day 5				Day 6				Day 7			
	N	I	M	D	N	I	M	D	N	I	M	D	N	I	M	D	N	I	M	D	N	I	M	D	N	I	M	D
S.S.-1	0	13	9	8	0	8	12	10	0	3	11	16	0	0	12	18	0	0	9	21	0	0	2	28	0	0	0	30
S.S.-2	0	22	3	5	0	9	15	6	0	6	9	15	0	0	9	21	0	0	3	27	0	0	0	30	0	0	0	30
S.S.-3	0	14	10	6	0	6	15	9	0	3	18	9	0	0	10	20	0	0	7	23	0	0	3	27	0	0	0	30
J.C.-1	0	4	20	6	0	3	12	15	0	0	6	24	0	0	6	24	0	0	0	30	0	0	0	30	0	0	0	30
J.C.-2	0	0	27	3	0	0	16	14	0	0	9	21	0	0	3	27	0	0	0	30	0	0	0	30	0	0	0	30
J.C.-3	0	3	18	9	0	2	14	14	0	0	4	26	0	0	0	30	0	0	0	30	0	0	0	30	0	0	0	30
Control	30	0	0	0	30	0	0	0	30	0	0	0	30	0	0	0	30	0	0	0	29	1	0	0	29	1	0	0

N = normal, I = intoxicated, M = moribund, D = dead

S.S = Sitka spruce, J.C. = Japanese cypress

6.5 Discussion and conclusion

The traditional method of drywood termite treatment involves either fumigation with sulfuryl fluoride or spot treatment using insecticide injection. Both approaches are the most widely used and most effectively control the infestation of drywood termite; however, their toxicity for the environment and human habitat and risk of re-infestation must be considered. The ability to control drywood termite infested structural timber by the two-in-one bandage system provides a composition comprising remedial treatment using carbon dioxide snow and a preventive treatment using imidacloprid.

Our assessment of the effectiveness of the remedial treatment in a two-in-one bandage system was highly influenced by the microstructure of the wood and the dosage. As a microstructural influencing factor, our results indicate that the thermal conductive properties of wood at low temperatures are strongly affected by the anisotropy nature of the wood (i.e., growth ring/grain direction, and the proportion of springwood and summerwood). A steep angle in Sitka spruce provides an indication of the radial direction, and a gentle angle in Japanese cypress provides a sign of the tangential direction. The few reports published on thermal conductivity of wood in both high and low temperatures have described that the thermal conductive properties in the radial direction are greater than in the tangential direction (Choi and Yoshimura, 2016b; Leon et al., 2000; Niemz et al., 2010; Wagner et al., 1994). Another indicator of the thermal conductive properties of wood can be determined from the proportion of spring- and summerwood. Below the fiber saturation points, larger cell lumens with thin walls of springwood are mostly filled with air, which has lower thermal properties than wood (Asako et al., 2002). In contrast with springwood, thick cell walls with smaller lumens increase the thermal properties of wood due to a lower percentage of air, consequently, the proportion of summerwood considerably affects the thermal conductive

properties of wood. The difference of springwood and summerwood of Norway spruce differ by three factors (Sonderegger et al., 2011).

Regarding the effectiveness of the remedial treatment at the highest dosage tested, 104.6 kg m^{-3} , the core temperature of timber achieved a lower than required critical thermal minimum ($CT_{\min}$) of drywood termite in the first 3 h, which resulted in 100% mortality in artificially infested structural timbers after a 20 h application. Based on the $CT_{\min}$ data in lower dosages (69.8 kg m^{-3} , 87.2 kg m^{-3}), the eradication of a colony is now likely to be achieved, however, these dosages would be effective for achieving a satisfactory level of mortality since the drywood foragers have a tendency to construct their chambers and superficial longitudinal expansions less than 30 mm beneath the surface of the wood (Choi and Yoshimura, 2016c). After the remedial treatment, we inspected the physical damage by the application, but there were no visual signs of damage on the artificially infested timbers. The freezing treatment to control drywood termites inside a wooden structure was conducted by a number of researchers using liquid nitrogen. Based on published reports, the minimum dosage required to achieve extermination of drywood termites with liquid nitrogen was 202 kg m^{-3} in an uninsulated wall (Rust et al., 1995b) and 381.8 kg m^{-3} in a building structure (Lewis and Haverty, 1996). Consequently, the remedial treatment in two-in-one bandage system is effective against drywood termites in a laboratory test.

Imidacloprid claims to be a slow-acting insecticide that is non-repellent and has been investigated extensively for soil treatment against subterranean termites (Baskaran et al., 1999; Osbrink et al., 2005; Thorne and Breisch, 2001). It has been suggested as a potentially useful tool for the protection of structural timber (Ahmed et al., 2004; Smith and Wu, 2005) and tree pests (Grosman and Upton, 2006). For the protection of structural timber, surface application is the simplest treatment to apply the insecticide to the wood by spraying, dipping or soaking, which produces

either a layer that prevents pest attacks or diffuses through the wood. The surface treatment enables restricted transverse penetration (less than 2.5 mm) below the surface of the timber (AWPA, 2007). After 12 h of the envelope treatment with and without carbon dioxide snow in our study, the transverse penetration of active compounds was obtained between 1.3 and 3.8 mm. The anatomical structure and MC of wood have an influenced on the high variation of transverse penetration (Lehringer et al., 2009).

The surface deposit layer of the active ingredient was not estimated in this study. However, the insecticidal activity evaluations performed in our study demonstrated that imidacloprid was effective in all three concentrations. The difference in the mortality data in this study indicates that there was a significant interaction between the dosage and the exposure time. With continuous exposure at the lowest concentration, worker health may not suffer significantly due to a delayed avoidance response of the termites (Grace, 1991). After day 1, we observed that termites stopped any foraging activity on the treated surface, but dead workers were not observed until day 3. Once workers started dying, the mortality rate increased because imidacloprid interferes with acetylcholine receptors in the insect's nervous system (Ramakrishnan et al., 1999), which accumulates the damage and reinforces the toxic effects of long exposure (Tennekes, 2011). The toxic effects of imidacloprid against drywood termites and the long-delayed toxicity of imidacloprid deposit against drywood foragers are explained by Rust and Venturina (2009), who reported that a 0.05% imidacloprid foam was able to terminate drywood termites after prolonged continuous exposure. Meanwhile, with continuous exposure at higher concentrations, termites were overwhelmed by the excessive amounts of imidacloprid, which required more resources and time to cope with the stress, enhancing the release of liquid excrement and proctodeal fluid (Quarcoo et al., 2010). Recognition of advanced symptoms induced by an insecticide are indicators

that insects are exposed to a sublethal dosage of insecticide (Weiss, 1988). Although all three dosages of imidacloprid were effective against drywood termites, the higher dosage is recommended for application due to the mode of action of imidacloprid.

The composition of the two-in-one remedial bandage system can be used to increase the life of structural timber in residential buildings materially. Our study has described the two-in-one remedial bandage system as a system that includes a remedial treatment using carbon dioxide snow and a preventive treatment using a non-repellent insecticide, imidacloprid, which is substantially capable of eradicating and protecting timber from re-infestation. However, this application is suitable for above-ground applications in the US (Freeman et al., 2006) and Japan (Tsunoda et al., 2004) and is a hazard class H2 by the Australian standard (Day et al., 2011).

This study was a test of new application efficacy under controlled conditions. Due to the controlled condition and the adoption of the latest monitoring technology of infestation, we obtained a high efficacy rate for the two-in-one remedial bandage system. However, the introduction of the two-in-one remedial bandage system as a pest management industry application requires that our research continues to improve and enhance the remedial bandage system and our understanding of the efficacy of carbon dioxide snow and other non-repellent insecticides

CHAPTER 7: An overall summary and Future Works

7.1 An overall summary

With current pest management industry standard for detection/inspection (visual searching) and remedial treatment (wood injection), it is hard to prevent drywood termite infestations for some reason including; 1) the lack of knowledge of foraging behavior and biological adaptations of drywood termites that involve survival in environmental stresses and 2) the high proportion of remedial intervention which only intends to kill drywood termites present at the time of application, whereas no preventive treatment which intends to stop or prevent reinfestation. Consequently, the drywood termite management should understand the foraging behavior and biological adaptations of drywood termites to provide a framework for improving efforts at detection and control when treatment is necessary. By extension, improvement and developments in the uses of remedial and preventive treatment which all aimed at minimizing detrimental side effects to structure and the environment.

This dissertation described two-in-one bandage system for improving remedial and preventive treatment of drywood termite control. The two-in-one bandage system is the result of a nearly four-year investigations on an understanding of the foraging behavior and biological adaptations of drywood termites, as well as design and implementation effort, focusing on the developing an efficient and useful application system to improve control of drywood termites in local infestation and provide a competitive cost advantage to pest management companies and property owners.

With the development of verifiable detection technology, numbers of studies revealed the foraging and nesting behavior of drywood termites that help to understand the feeding ecology. According to the CT scanning results obtained in Chapter 2, drywood termite foragers have

distinguished foraging patterns based on the physical properties of wood during gallery construction, and foragers primarily excavate along the springwood (radial pattern) towards the outer surface of the wood to establish the primary and satellite chambers, followed by mainly superficial (0.3 — 3.0 cm under the surface) longitudinal expansion, and occasional lateral direction expansion. The development of tunneling is effective by the physical constraints of knots (high lignin content) and upsets (high extractive content). These unique foraging behavior of drywood termites aids the development and optimization of the application of new remedial treatment using solid carbon dioxide as a chilling agent.

Publications in Chapter 3 and 4 add on to a literature developed in the 1990s and 2000s dealing with the response of biological adaptations of termites to the environmental stress (desiccation and temperature). The mode of action in killing termite using lethal low temperature involves the formation of ice crystals in the hemolymph that destroys cellular membranes give rise to the death. Therefore, termites regulate the body temperature by the temperature of the environment evolving a diversity of competitive biological adaptation to ensure the survival under environmental stresses (temperature and desiccation) in nature. For subterranean termite species, behavioral avoidance of low temperatures is more important than increases in physiological cold tolerance. As a classic example of habitat exploration of *Reticulitermes speratus* spp., they detect seasonal changes in soil and relocate to the deeper ground or to heated structures in an urban habitat in which the temperature is buffered from the climate. Whereas, the refugia for drywood termites to relocate to are limited when ambient conditions become unfavorable since the galleries of drywood termites are within the structures of buildings with no contact to soil. Therefore, a reasonable assumption is that the desiccation and temperature tolerance of *I. minor*, are likely greater than those of subterranean termite species. In Chapter 3, we examined biological adaptations in physiological

elements such as water relations, carbohydrate metabolites, and cuticular structure to determine how *I. minor* withstands low temperature stress and the results show that cold tolerance of *I. minor* was influenced by some physiological elements that included low body water content and hardened structure of the cuticle (cuticle thickness, cuticular lipid content, and long-chain hydrocarbons). Although trehalose is a cryoprotectant in one-piece-type (use the same piece of wood as their shelter and food) termite species, the result of this study indicated that trehalose did not function as a natural cryoprotectant in *I. minor*. Consequently, the author was anxious about the key factors of physiological elements of termites against environment stresses (desiccation and temperature), and the studies have been conducted, and the results of these studies were presented in Chapter 4a and 4b. There are a remarkable number of studies indicated that there is a degree of cross-tolerance between desiccation and low temperature. As a general rule, insects live in the arid/xeric environment have evolved to tolerance than those living in mesic/hydric environments. Various researches have been studied that drywood termites have developed several competitive biological adaptations to withstand extremely temperature fluctuations and xeric habitats. Most stand-out developed biological adaptations are cuticle structure (Hadley, 1994; Woodrow et al., 2000) and regulating of body water content (Cabrera and Rust, 1996; Steward, 1982; Steward, 1981). In comparison with other termite species, drywood termites consist thicker cuticle (Minnick et al., 1973), and the larger proportion of saturated cuticular hydrocarbons of *I. minor* indicated the greater the effectiveness of the cuticular lipids in preventing desiccation (Collins and Richards, 1966; Howard et al., 1978). As demonstrated in numerous comparative studies of adaptations, the results from Chapter 4a show that biological adaptations (i.e., a relatively large body size, a large mass of reserves that are metabolized by reducing body water mass, a mass of cuticular lipids, and cuticular hydrocarbons) influenced the cuticular resistance in desiccating conditions. From the

analysis of the chemical taxonomy of cuticular hydrocarbons (CHC), which are often exposed to a desiccating environment in termites, *I. minor* possessed greater quantities of saturated (long-chain) cuticular hydrocarbons which indicate the effectiveness of the cuticular lipids in preventing desiccation. As a finding of the study on the correlation between biological properties and desiccation tolerance was that carbohydrate metabolites (trehalose) did not function as the main shield for *I. minor*. Another finding from the survival of drywood termite under induced high carbon dioxide and desiccated atmosphere was that the resistance to desiccation in drywood termites is not solely a function of physiological adaptation, but the additional mechanism (adaptive behavior: social aggregation, reducing locomotion) that ensure the survival of the entire colony survival under desiccation. Another objective of Chapter 4a was to investigate the immediate effects of an atmosphere with experimentally induced high levels of carbon dioxide by sublimation of solid carbon dioxide on the dehydration of termites. In an artificially induced high CO₂ atmosphere, the loss of body water from drywood termites increased, which led to increased rates of mortality. Although the mechanism for the increased loss of body water under high CO₂ conditions was not determined, however, the recognition of advanced symptoms was noted from the groups of *I. minor*. Under CO₂ narcosis workers defecated on average 14–20 pellets (mean 0.91 mg; 1.0% of wet weight). In this scenario, CO₂ initially causes an oxygen deficit, and lactic acid is produced in the muscles; then, the high lactic acid content reduces the performance of muscles such as those of the termite anus, which leads to an increase in defecation.

In common freezing avoiding insects, the concentration of carbohydrate and polyols usually increase from autumn onwards. Moreover, insects in colder habitat contain a higher level of trehalose. Furthermore, insects living at higher latitudes tend to be more tolerant to low temperatures than their counterparts because temperature variation increases with latitude.

However, this hypothesis may not reflect the evolution of a suite of physiological adaptation in drywood termites. First, western drywood termite is native to the desert and semi-arid southwestern United States. When it is invasive, it seems to be concentrated in air-dried timbers in hot and dry areas of buildings. Second, the current distribution of *I. minor* is mainly limited to the coastal area (Humid subtropical climate) of Japan. This null hypothesis has led to additional studies seeking to understand the mechanisms of cold tolerance of *I. minor* by comparing the physiological elements of *R. speratus* from three contrasting locations (Kagoshima, Kyoto, Sapporo) in Chapter 4b. The results of Chapter 4a showed that carbohydrate metabolites (glucose, trehalose) and balancing of total body water and total lipids accumulate in *R. speratus* with a substantial decrease in temperature, with the effect being a slight enhancement in the cold tolerance of the species in all three contrasting locations. Within three contrasting locations, *R. speratus* populations in Kyoto had higher levels of carbohydrate metabolites alteration, lower supercooling points, and lower lethal temperatures. The results of this study support the null hypothesis that carbohydrate metabolites (trehalose) did not function as a key role in the order of Isoptera as a physiological adaptation that ensures the survival under sub-optimal temperature. Collected metabolites and thermal response data suggest that the low body water content and harder cuticle structure (i.e., a higher concentration of cuticular lipids and higher molecular weight and long-chain compounds) may influence on cold tolerance.

To develop and optimize the new remedial treatment, the knowledge of the thermal conductive properties of wood and heat transfer properties of solid carbon dioxide is another important subject which needs to be investigated. Several studies have reported that high efficacy of lethal low temperature remedial treatment using liquid nitrogen under laboratory and field conditions. However, none of these studies investigated the relationship between the thermal conductive

properties of wood at low temperature and the various factors affecting these properties. The results in Chapter 5 indicate that the thermal conductive properties of wood at low temperatures are mainly affected by the composition and morphological properties of wood (i.e., density/ratio of earlywood and latewood, the proportion of heartwood and sapwood, the pattern of growth ring). In particular, thermal conductive properties are mainly dependent on the pattern of the growth ring itself at low temperatures. In a comparison of two different solid carbon dioxide forms, it appears that crushed small particles/snow solid carbon dioxide increases the thermal conductive properties of the wood species tested. This phenomenon might be caused by the faster sublimation of small particle/snow which extracts the heat energy from the immediate surroundings resulting in extreme cooling compared with solid carbon dioxide slabs.

The first investigation on using a lethal cold temperature treatment for drywood termite control was a study applying liquid nitrogen into wall voids to create temperatures below the critical thermal minimum of drywood termites (Forbes and Ebeling, 1986), however, it is not known if the use of liquid nitrogen to control drywood termite. Drywood termite control applications relying on a single treatment remain a suboptimal method since complete protection is not possible in thermal applications because there is no residual protection. Consequently, the approach of a combination of two or more treatments with different mechanisms can cooperatively prohibit drywood termite eradication and prevent re-infestation. Publication in Chapter 6 presents the development of a two-in-one drywood termite control application that evolves into a remedial treatment using carbon dioxide snow and preventive treatment, imidacloprid. Similar to the result from Chapter 5, the effectiveness of the remedial treatment in a two-in-one bandage system was highly influenced by the microstructure of the wood and the dosage of the chilling agent. However, using polyurethane bandage system requires a much less chilling agent (1,200g carbon dioxide snow) to generate the

core temperature of timber achieved a lower than required critical thermal minimum (CT_{min}) of drywood termite in the first 3 h in the much larger volume of structural timber (0.0115 m^3 of softwood). It explains the importance of designing and material selection for the insulation system to maximize the heat transfer properties of solid carbon dioxide. As a preventive treatment, the insecticidal activity evaluations performed in this study demonstrated that imidacloprid was effective in all three concentrations. The difference in the mortality data in this study indicates that there was a significant interaction between the dosage and the exposure time. With continuous exposure at the lowest concentration, worker health did not suffer significantly due to a delayed avoidance response of the termites. Meanwhile, with continuous exposure at higher concentrations, termites were overwhelmed by the excessive amounts of imidacloprid, which required more resources and time to cope with the stress, enhancing the recognition of advanced symptoms.

7.2 Future works

This dissertation works investigated on the foraging behavior and biological adaptations of drywood termites to provide a framework for improving efforts at control when treatment is necessary. It has been verified that the properties of cuticle have great impact on the resistance against environment stresses. Therefore, more research is needed to determine the function of cuticle on the influence factor against cold temperature tolerance.

This dissertation has presented a two-in-one bandage system, which combines of two or more treatment approaches with different mechanisms can cooperatively prohibit drywood termite eradication and prevention. Although this study shows promise for improved methods of control and prevention for drywood termites, there is potentially adjusting this new control technique/device to meet the needs of the pest management industry. Area of potential future works is to develop the supercritical mixture of the chilling agent and insecticide. Using the

supercritical CO₂ mixture, it would reduce the application time and cost for the pest management industry. In the current design, the increase the property of insulation of two-in-one bandage system by the implement of other insulative materials.

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