EFFECTS OF EXTREME TEMPERATURES ON THE SURVIVAL OF THE QUARANTINE STORED-PRODUCT PEST, *TROGODERMA GRANARIUM* (KHAPRA BEETLE) AND ON ITS ASSOCIATED BACTERIA

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ABSTRACT

Trogoderma granarium is a pest of stored-grain products in Asia and Africa, and a quarantine pest for much of the rest of the world. To evaluate extreme temperatures as a control strategy for this pest, I investigated the effect of high and low temperatures on the survival (Chapters 2 and 3) and on the microbiome (Chapter 4) of *T. granarium.* The most cold- and heat-tolerant life stages were diapausing-acclimated larvae and diapausing larvae, respectively. *Trogoderma granarium* can be controlled (Probit 9) with an exposure of 70 d to −15°C or 1.2 h to 60°C. High temperatures affected the microbiome; an effect of low temperatures was not detected. While the microbiome changed with life stage, it was dominated by *Spiroplasma* bacteria. Further research is necessary to understand the *Spiroplasma-T. granarium* relationship. Future research should also investigate combinations of extreme temperatures with other techniques to shorten the time required for mortality.

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LIST OF ABBREVIATIONS

Symbol	Definition
R.H.	Relative humidity
SCP	Supercooling point
hsps	Heat shock proteins
PCR	Polymerase chain-reaction
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
NGS	Next-generation sequencing
LT	Lethal time
wk	week
d	day
h	hour
min	minute
g	grams
DL	Diapausing larvae
OTU	Operational taxonomic units
bp	Base pairs
PCoA	Principal coordinate analysis
H’	Shannon index
GLM	Generalized linear model

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW [*]

OBJECTIVES

This thesis seeks to evaluate the use of extreme temperatures, and the factors influencing their application, as a control method for khapra beetle, *Trogoderma granarium*. The two main goals are to:

1) Assess use of extreme temperatures to kill *T. granarium*. Specifically, to determine the duration of exposure at different temperatures (high, low) needed to kill all life stages of *T. granarium* (egg, larva, diapausing larva, cold-acclimated larva, pupae, and adult). The assessment of the use of extreme temperatures to control *T. granarium* addresses a need to replace current chemical methods of control.

2) Characterize the community of symbiotic bacteria associated with *T. granarium* and how it is affected by extreme temperatures. The assessment of *T. granarium* microbiome can provide new insights into the development or improvement of the use of extreme temperatures and other methods of control for this pest.

LITERATURE REVIEW

This study focused on khapra beetle (Coleoptera: Dermestidae), *Trogoderma granarium* Everts. I examined *T. granarium* tolerance to extreme temperatures, its associated bacterial community, and the effects of extreme temperatures on the associated bacterial community. This literature review will first review general information about the family Dermestidae, specifically *T. granarium*, and the strategies currently used for its control.

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Second, it presents the use of extreme temperatures as a method of control, the mechanisms of insect tolerance to extreme temperatures, the factors that affect temperature tolerance, previous data on control of dermestids at low and high temperatures, and the application of these data for the development of mathematical models to control pests with extreme temperatures. Third, it presents the concept of the microbiome and its importance for pest control, the effects of the microbiome on temperature tolerance and the effects of temperatures on the microbiome, previous information about the microbiome of stored-product pests and *T. granarium*, and the methodological approaches used to study the insect microbiomes.

1. Study organism

Dermestid beetles comprise a diverse group of more than 1200 described species in 45 genera. Most species are xerophilous necrophages; i.e., they develop on the desiccated tissues and hairs of dead animals (Háva & Nardi, 2004, Kiselyova & McHugh, 2006, Zhantiev, 2009) (Table 1.1). Necrophagous species (e.g., *Dermestes* spp.) feed in animal carcasses and on dead insects (Kingsolver, 2002, Zhantiev, 2009). Nidicolous species (e.g., *Anthrenus* spp.) occur on mammals and in bird nests, where they feed on hair or feathers (Peacock, 1992, Háva, 2004, Zhantiev, 2009). A small number of species are predators that feed on wasp and bee larvae, and on spider eggs (Zhantiev, 2009). Feeding on plants is atypical for the family, but their tolerance to low humidity allows some dermestids (mainly *Trogoderma* spp. and *Attagenus* spp.) to develop solely on dried cereal products (Hinton, 1945, Kiselyova & McHugh, 2006, Zhantiev, 2009).

A number of dermestid species are of key economic importance. Some genera (*Trogoderma*, *Attagenus*) are pests of stored grains and stored grain products (Hinton, 1945, OEPP/EPPO, 2013). Other genera (*Attagenus* spp., *Anthrenus* spp.) are common in museums, where they damage preserved insect, bird, and mammal specimens, or items that contain plant or animal materials (Pinniger, 1991, Veer *et al.*, 1991, Linnie, 1994). Species of *Anthrenus*, *Anthrenocerus* and *Dermestes* develop in products that contain wool, cotton, linen, synthetic fibres, and silk (Bennett *et al.*, 1988, OEPP/EPPO, 2013). Species of *Attagenus* and *Dermestes* consume silkworm cocoons and prey on different life stages of silkworms (Kumar *et al.*, 1988, Veer *et al.*, 1996). About 28 species of *Dermestes* infest stored silkworm cocoons and *D. maculatus* is a reported vector of the microsporidian *Nosema bombycis* Nageli, which is the causative agent of pébrine disease in silkworms (Veer *et al.*, 1996). Dermestids also infest the packaging and containers used to ship stored products which, in combination with international commerce, have contributed to the cosmopolitan distribution of many dermestid species (Wildey & Wayman, 1979, Turner, 1986).

Khapra beetle, *T. granarium*, is among the most important invasive species and pests of stored agricultural products (Eliopoulos, 2013). It is native to India, but due to international trade, it is currently present in Europe, North Africa, South Asia, and the Middle East (Hagstrum & Subramanyam, 2009, Eliopoulos, 2013). In these countries, it has been found infesting 96 commodities, with a preference for grains, cereals, and spices (Hagstrum & Subramanyam, 2009). It is a quarantine insect for Canada, United States, South America, Australia, and New Zealand (OEPP/EPPO, 2013, OEPP/EPPO, 2015). If introduced into these countries, it would cause a high economic impact (Eliopoulos,

2013); countries with infestations of *T. granarium* have limited access to international markets (Myers & Hagstrum, 2012, Eliopoulos, 2013). The high importance of *T. granarium* worldwide lies not only in the damage it can cause to a wide variety of commodities, but also in that it can get established in almost any climate in closed storage facilities (Lindgren & Vincent, 1959). The capacity of its larvae to diapause allows it to tolerate high doses of insecticides and extreme conditions (Lindgren & Vincent, 1959, Bell *et al.*, 1984). For example, it has the ability to survive without food for long periods of time, and to survive and reproduce at 0% relative humidity (R.H.) (Lindgren & Vincent, 1959, Eliopoulos, 2013).

All life stages of *T. granarium* are resistant to heat and dryness. The optimal conditions for its development are 30-37^{o}C and 40-60% R.H. at which the life cycle from egg to adult is completed from 24-36 days (Table 1.1) (Hinton, 1945, Hadaway, 1956, Burges, 1962a, Yinon, 1968). At temperatures of 10-20^{o}C, *T. granarium* survives, but does not mate nor lay eggs (Hadaway, 1956).

2. Control of *Trogoderma granarium*

Given the economic importance of *T. granarium*, there is a large body of research describing measures for its control. Control, however, is complicated by the ability of this insect to undergo diapause. This ability is uncommon among stored-product insects and increases their tolerance to extreme climatic conditions and insecticides (Bell, 1994).

Control primarily has relied on fumigants and contact insecticides. Fumigants that have been used for control include methyl bromide (Lindgren & Vincent, 1959, Linnie, 1994, MBTOC, 2014, Fields & White, 2002), phosphine (Vincent & Lindgren, 1972, Bell *et al.*,

1984, Linnie, 1994, Udeaan, 1990, Bell & Wilson, 1995, Ahmedani *et al.*, 2007, Ahmad & Sarfraz, 2000), and sulfuryl fluoride (Su & Scheffrahn, 1990, Rajendran *et al.*, 2008, Sriranjini & Rajendran, 2008). Contact insecticides used for control include malathion (Lindgren & Vincent, 1959, Singh & Yadav, 1994, Khosla *et al.*, 2005), and chlorpyriphos (Singh & Yadav, 1994, Khosla *et al.*, 2005, Eliopoulos, 2013). Frequent use of fumigants and contact insecticides may cause the evolution of resistance, introduces residues into the environment, and can be detrimental to consumer health (Cao *et al.*, 2002, Rajendran, 2002, Rajendran & Hajira Parveen, 2005). Methyl bromide is an ozone-depleting substance. In accordance with the Montreal Protocol, its use has been generally banned on a global basis with exemptions for quarantine uses (MBTOC, 2014). Phosphine is less effective than methyl bromide and some strains of *T. granarium* have evolved phosphine resistance (Vincent & Lindgren, 1972, Bell *et al.*, 1984, Alam *et al.*, 1999, Rajendran, 2002). These issues have led to examination of non-chemical methods of control, depending with the nature of the infested product (Rajendran & Hajira Parveen, 2005). Alternative methods include the use of controlled atmosphere (CO_2-rich, O_2-poor atmospheres) (Khatoon & Heather, 1990, Reichmuth *et al.*, 1992), plant extracts (Traynier *et al.*, 1994, Sharma *et al.*, 2015), inert dust (Nakamoto, 1989), radiation (Khatoon & Heather, 1990), and extreme temperatures (Fields & White, 2002, Wright, 2002, Abdelghany *et al.*, 2015).

3. Extreme temperatures

The use of extreme temperatures may provide the most viable alternative to chemical applications. It has been used as an insect-control method in North America since the early 1900s (Hansen *et al.*, 2011, Mathlein, 1961, Burges & Burrell, 1964). There are no

reported cases of insects developing resistance to heat or cold, there are no residues to harm consumers, and it does not require registration, unlike the use of insecticides in most jurisdictions (Fields & White, 2002, Eliopoulos *et al.*, 2011, Fields *et al.*, 2012). However, adoption of extreme temperatures as a treatment method has been hampered by an inability to increase or decrease temperatures economically and quickly, as well as by a lack of knowledge on the combination of temperature and period of exposure that is needed for control (Strang, 1992, Bergh *et al.*, 2006). Some of the studies that report on the thermal-mortality limits are based on observation rather than on controlled experiments (Linnie, 1999). Furthermore, much of the literature on the temperature tolerance of dermestids is difficult to access or not recent (Fields, 1992, Linnie, 1999).

3.1 Mechanisms of temperature tolerance

Temperature influences almost every aspect of insect biology. Temperature affects metabolic rates, defines limits of physiological function, determines developmental times, impacts behaviour, and ultimately influences survival of the population (Lee, 1991, Bhargava *et al.*, 2007). For stored-product insects, optimal temperatures maximize fitness and population growth. Stored-product insects require a high minimum temperature before they can complete their development. In general, temperatures above 15^{o}C are needed for egg-laying and optimal temperatures will range between 25-35^{o}C (Fields, 1992, Strang, 1992). Suboptimal temperatures (35-40^{o}C and 13-25^{o}C) allow completion of life cycles and reproduction, but populations merely persist rather than thrive. Lethal temperatures (above 40^{o}C and below 13^{o}C) eventually cause population extinction (Fields, 1992, Wellheiser, 1992, Bhargava *et al.*, 2007). Different species can have slightly different temperature responses (Hadaway, 1956, Loschiavo, 1960, Coombs,

1981, Burges, 2008, Riaz *et al.*, 2014). Cold and heat-tolerance refers to the capacity of an organism to survive or tolerate exposure to temperatures and exposure times that are otherwise suboptimal or lethal (Lee, 1991).

3.1.1 Mechanisms of cold tolerance

Exposure to cold can damage insect cell membranes, cause the loss of ion homeostasis, reduce metabolic rates, and mechanically damage tissues (Fields, 1992, Lee, 2010). In general, insects are classified as freeze-intolerant (die when frozen) or freeze-tolerant (capable of surviving ice in their bodies) (Fields, 1992, Lee, 2010). Other systems of classification have been proposed that take into consideration the different aspects of cold mortality, but these have not been widely adopted (Nedved, 1999). All dermestids and other stored-product or museum-collection insect pests studied to date are freeze-intolerant, and die at their supercooling point (SCP), which in general range from −10 to −20^{o}C (Evans, 1986, Strang, 1992, Fields *et al.*, 1998, Linnie, 1999, Fields, 2001, Fields *et al.*, 2012). There is significant mortality at temperatures warmer than the SCP, with warmer temperatures causing less mortality (Fields, 1992, Strang, 1992).

3.1.2 Mechanisms of heat tolerance

Exposure to sublethal high temperatures increases metabolic rates followed by the cessation of movement in some insects. Prolonged exposure can change the fluidity of cell membranes, alter ionic balance, and alter the structure of macromolecules to affect cell structure and the rate of metabolic reactions (Fields, 1992, Nedved, 1999, Hansen *et al.*, 2011). High temperatures can also increase the permeability of the cuticle wax complex causing water loss and desiccation (Nedved, 1999). Heat damage is normally

irreversible and, even if the insect survives heat exposure, it may fail to complete development or reproduce when returned to optimal temperatures (Fields, 1992, Denlinger & Yocum, 1998, Nedved, 1999, Hansen *et al.*, 2011). To achieve heat tolerance, heat shock or stress proteins replace the production of normal proteins, which reduce the damage to susceptible proteins (Denlinger & Yocum, 1998). Previous studies show that all life stages of dermestids and other stored-product insects can survive at 38^{o}C, but most of them will be killed by an exposure of 2 hours at 50^{o}C (Michelbacher, 1953, Lindgren & Vincent, 1959, Evans, 1986, Fields, 1992, Phillips & Throne, 2010, Fields *et al.*, 2012).

3.2 Factors affecting thermal tolerance

Tolerance to extreme temperatures is highly variable within and among species due to multiple factors (Lee, 1991, Fields, 2001). Identifying these factors is critical when designing thermal treatments for the control of pest insects, particularly those life stages and species that are most tolerant (Beckett *et al.*, 2007). Factors that affect temperature tolerance include: i) life history of the species and symbiosis with microorganisms, ii) stage of development, iii) diapause, iv) relative humidity, v) acclimation, and vi) temperature and duration of exposure (Salt, 1958, Evans, 1986, Lee, 1991, Fields, 1992, Fields, 2001, Beckett *et al.*, 2007).

3.2.1 Life history of the species and symbiosis with microorganisms

The environmental conditions normally experienced by a species often define its thermal tolerance (Chown, 2001). For example, insects not normally found at cold temperatures usually cannot tolerate freezing temperatures and may die after even a brief exposure to

suboptimal temperatures (Salt, 1961, Doucet *et al.*, 2009). Most granivorous species are tropical or sub-tropical species able to breed rapidly over a fairly wide range of temperatures (Burges & Burrell, 1964). In general, however, they are more susceptible to low temperatures compared to species of insects from temperate and cold zones (Burges & Burrell, 1964).

Species association with microorganisms (symbiosis) can affect the tolerance of the insect species to extreme conditions; e.g., temperatures (Douglas, 2007, Zindel *et al.*, 2011, Wernegreen, 2012). Symbiotic microorganisms influence various traits of the biology and ecology of their host, and there are multiple examples where these relationships increase or decrease the host tolerance to high and low temperatures (Wernegreen, 2012) (see section 5).

3.2.2 Stage of development

Thermal tolerance differs among developmental stages mainly due to their physiological differences (Salt, 1958, Fields, 1992, Danks, 1996, Danks, 2005, Danks, 2006, Beckett *et al.*, 2007). Life stages of smaller size and lower water content have lower supercooling points. The digestive tract of non-feeding life stages (e.g., pupae) do not contain food particles (which can act as ice-nucleators) and, therefore, have lower SCPs than feeding stages (Denlinger, 1991, Lee, 1991). Different life stages also may be associated with different microhabitats, which can affect SCPs, freezing capacity, and loss of water (Denlinger, 1991, Fields, 1992). A lower SCP does not necessarily mean a higher cold-tolerance; for example, eggs often have low SCP but are not the most cold-tolerant stage

(Fields, 1992). However, within a given life stage, SCPs can often be correlated with cold tolerance (Fields, 1992, Abdelghany *et al.*, 2015).

3.2.3 Diapause

Diapause is an endocrine-mediated dormancy characterized by an arrest in development and suppression of metabolism that may be either obligate or facultative (Denlinger, 1991, Bell, 1994). For insects with facultative diapause, diapause is initiated, maintained, and terminated by external cues. Thus, depending upon environmental conditions, a given generation of an insect may or may not enter diapause (Tauber *et al.*, 1986, Koštál, 2006). Nair and Desai (1973b) showed that strains of *T. granarium* where facultative diapause was induced by crowding and prevented by isolation could be selected so that there was no diapause under crowded conditions. In other words, they produced a non-diapausing strain. Also a strain could be selected so that almost all larvae in both crowded (diapausing) and isolated (non-diapausing) conditions entered into diapause; i.e., they produced a strain with obligate diapause. Their finding suggests that diapause is determined by multiple genes (Burges, 1962a, Nair & Desai, 1973a). It also indicates that laboratory strains could lose the ability to diapause depending upon the method of rearing and number of generations in culture. If time is a confounding factor, tests performed over several generations on the characteristics of diapause (induction, maintenance and termination) may lead to erroneous conclusions if the correct controls are not present.

Insects entering diapause undergo physiological changes that enhance the accumulation of metabolic reserves and reduce rates of energy and oxygen consumption (Tauber *et al.*, 1986, Denlinger, 1991). This is achieved by the activation of diapause-specific genes that

encode for proteins, which affect heat shock, energy metabolism and storage, hormonal regulation, and clock proteins, that influence diapause induction (Koštál, 2006). Mechanisms for diapause-induction and cold tolerance may interact; e.g., both are regulated by the juvenile hormone and both include the production of cryoprotectants, elimination of gut contents, and dehydration of body tissues (Lindgren & Vincent, 1959, Bell *et al.*, 1984, Tauber *et al.*, 1986, Danks, 1996, Danks, 2005, Danks, 2006). However, the relation between diapause and cold-tolerance is not always clear. Both are dynamic processes and, even within a species, some elements of diapause and cold-tolerance are linked whereas others are not (Danks, 2006, Danks, 2005). Also, depending upon the species, the co-occurrence of diapause and cold-tolerance may be coincidental or reflect independent responses to similar inductive cues. In addition, cold-tolerance may occur in the absence of diapause (and vice versa) and may increase with acclimation (Tauber *et al.*, 1986, Denlinger, 1991, Danks, 2006, Danks, 2005).

Diapause can provide higher tolerance to detrimental conditions and can improve survival to extreme environments in some dermestids. The ability to undergo diapause as larvae evolved to tolerate suboptimal conditions where the food supply is sporadic and unpredictable. Because of this, the diapause stage allows higher survival in their ancestral environments as scavengers and in the invasion of a new niche as stored-product pests (Beck, 1973, Armes, 1988, Armes, 1990). Diapause allows individuals to synchronize development within populations and to survive conditions of extreme temperatures and desiccation, and may increase resistance to fumigants; e.g., phosphine and methyl bromide (Burges, 1962a, Tauber *et al.*, 1986, Pullin, 1996). When conditions become conducive for growth and reproduction, the diapause stage terminates, allowing for a

rapid increase in the population. For example, adult *T. granarium* that went through diapause are larger and laid more eggs than the ones that did not, promoting in that way a rapid increase of the population after diapause (Karnavar, 1972).

Most dermestids that diapause do so as larvae. Some have obligate diapause, while others have facultative diapause (Table 1.1). The genera *Anthrenus* and *Attagenus*, which are more closely associated with ancestral environments, have long life cycles (1-3 years) with a clear obligate larval diapause stage that enhances overwinter survival (Bell, 1994). For example, *Anthrenus verbasci* can have two periods of diapause, first as early instar larvae and second as mature larvae (Blake, 1959, Blake, 1958). *Anthrenus sarnicus* and *Anthrenus flavipes* undergo diapause as mature larvae only (Griswold & Greenwald, 1941, Armes, 1988, Armes, 1990). In these three species, diapause is controlled by an "internal clock"; all individuals go through diapause, but the timing and duration of diapause can be affected by photoperiod and temperature (Blake, 1959, Blake, 1958, Armes, 1988, Armes, 1990).

Facultative diapause is present mainly in *Trogoderma* spp. and is defined as a density-dependent phenomenon. During this stage individuals stay as mature larvae that feed and moult occasionally, and which can be identified by a low respiration rate (Beck, 1971a) and a longer larval stage (Burges, 1959a, Burges, 1960, Burges, 1962a, Burges, 1962b, Nair & Desai, 1973b, Nair & Desai, 1972, Nair & Desai, 1973a). This stage is induced by factors that can act alone or in combination, such as exposure to low temperatures, inadequate food, and rearing at low or high densities (Burges, 1962a, Nair & Desai, 1972). The diapause induced by larval crowding has been widely studied in *T. granarium*, but a similar facultative diapause has been described in *Attagenus elongatulus* (Barak &

Burkholder, 1977), *Attagenus unicolor* (Baker, 1977), and *Dermestes maculatus* (Osuji, 1975). This type of diapause is terminated by a rapid increase in temperature after exposure to low temperatures. When larvae undergo diapause as a result of crowded conditions, the renewal of food has little effect on termination and is effective only when accompanied by isolation (Burges, 1962b, Nair & Desai, 1973b). In contrast, larvae of *T. glabrum* and *T.variabile* have a facultative diapause defined as density-independent. The density-independent diapause is triggered by short photoperiods, low temperatures, small food volumes, isolation (not by crowding), or disturbance of the larvae (Loschiavo, 1960, Burges, 1961, Wright & Cartledge, 1994, Abdelghany *et al.*, 2015). The termination of density-independent diapause can be spontaneous or follow changes in density of larvae, a rapid increase in temperature, photoperiod, or the presence of pupae (Loschiavo, 1960, Burges, 1961, Beck, 1971b, Beck, 1971a, Wright & Cartledge, 1994).

The type of dormancy seen in dermestids has generated disagreement because it is different than in most insects (Tauber *et al.*, 1986, Bell, 1994). For example, larvae of *Trogoderma* spp. occasionally move, feed, and moult during dormancy (Burges, 1959a, Burges, 1960, Nair & Desai, 1972, Barak & Burkholder, 1977, Bell, 1994). Beck (1971a) suggested that these larvae should not be considered in diapause. Burges (1959a) questioned whether *T. granarium* is in quiescence or in diapause when dormant. Future studies should, in particular, identify triggers for induction and termination of diapause, and develop methods to better identify diapausing individuals.

3.2.4 Relative humidity

Dermestid beetles are xerophilous (thrive in dry environments) and are able to survive feeding on food with a water content below 10% (Zhantiev, 2009). This likely reflects the use of water obtained from metabolism, the absorption of water from the air, and (or) other strategies (Zhantiev, 2009, Edney, 1971).

Lack of water can also increase insect mortality at extreme temperatures (Convey, 1999, Everatt *et al.*, 2014). The mechanisms of injury caused by extreme temperatures and desiccation are similar in terms of osmotic stress and elicit similar responses for survival (Convey, 1999). Low relative humidity can reduce cold tolerance in other stored-product insects such as *Cryptolestes ferrugineus* (Fields *et al.*, 1998), *Oryzaephilus surinamensis* (Linnaeus), *Rhyzopertha dominica* (Fabricius), *Sitophilus granaries* (Linnaeus), *S. oryzae* (Linnaeus), and *Tribolium castaneum* (Herbst) (Evans, 1983, Fields, 1990). Similarly, at temperatures between 40-55^{o}C, low humidity (< 50% RH) greatly reduces survival (Fields, 1992, Beckett *et al.*, 2007), but humidity has less effect on mortality at higher temperatures.

3.2.5 Acclimation

Acclimation is a phenomenon whereby insect survival at extreme temperatures is enhanced by a brief exposure to less severe cold or high temperatures (rapid cold/heat hardening) (Denlinger *et al.*, 1991). It is associated with the expression of heat shock proteins or stress proteins (hsps), which protect organisms from heat/cold shock by stabilizing and preventing the aggregation, and improper folding of proteins (Denlinger *et al.*, 1991, Fields *et al.*, 1998, Beckett *et al.*, 2007). In insect pest-control applications,

there is little evidence suggesting that heat-acclimation has any practical significance at temperatures used for control, 50-60^{o}C (Evans, 1986). At low temperatures, rapid rates of cooling prevent acclimation, which otherwise can increase survival by two- to ten-fold (Evans, 1986, Fields, 1992, Beckett *et al.*, 2007). For *T. variabile*, cold-acclimated diapausing larvae are much more cold-tolerant than non-acclimated diapausing larvae (Abdelghany *et al.*, 2015).

3.2.6 Temperature and duration of exposure

The level of mortality experienced by insects at a given temperature is a function of the duration of exposure (Salt, 1958, Fields, 1992, Strang, 1992, Linnie, 1999). Normally, the relationship is determined experimentally to calculate the combination of temperature and duration needed to kill a given percentage of the population (Fields, 1992, Fields & White, 2002, Eliopoulos *et al.*, 2011). In *T. variabile*, 100% of non-diapausing larvae are killed after 17 days of exposure to -5^{o}C or after 1 day at -20^{o}C (Table 1.2) (Abdelghany *et al.*, 2015).

4. Control of dermestids with extreme temperatures

Extreme high and low temperatures are becoming more widely used to eradicate infestations of dermestids in storage facilities and museum collections (Bergh *et al.*, 2006). However, most dermestid species can enter diapause (Table 1.1), which may increase their tolerance to extreme temperatures (Bell, 1994, Fields, 1992). Thus, successful application of extreme temperatures requires information on the mortality of given species under different combinations of temperatures and durations, and consideration of how diapause and acclimation affect thermal tolerance (Burges &

Burrell, 1964, Strang, 1992, Fields, 2001). In the following sections, in order to establish a frame of temperatures to control *T. granarium*, I briefly report on the literature pertaining to the thermal tolerance of dermestids.

4.1 Low temperature

I identified 23 papers pertaining to the survival of economically-important dermestid beetles at low temperatures (Table 1.2). Seven of these were published since 2005; the remainder were published prior to 1980. Most of the information about control of dermestids with low temperatures is on *T. granarium* larvae held at −10°C. Depending upon the study, mortality of > 95% is achieved after 30 d of exposure (Mathlein, 1961, Lindgren & Vincent, 1959), of 91% after 72 h (Reynolds & Rundle, 1967), or 73% (Hinton, 1945) or 50% (Strang, 1992) after 25 h. This variation may be due to use of different methods (Fields, 1992, Zhang, 2012). Many studies provide little or no information on whether larvae were acclimated or in diapause, how the data were analysed, or on confidence limits to assess the variation in responses among individuals.

Only six studies have assessed the effect of acclimation and diapause on dermestids' cold-tolerance. Lindgren and Vincent (1959) showed that starvation reduced the mortality of *T. granarium* larvae held for 36 days at −10°C (Table 1.2). These starving larvae were likely in diapause (Burges, 1960). Three studies report an acclimation protocol before exposing the larvae of *T. granarium* (Hinton, 1945), *T. inclusum* (Reguzzi *et al.*, 2011), and *T. variabile* (Abdelghany *et al.*, 2015) to sub-zero temperatures. Results of these studies indicate that acclimation enhances survival at low temperatures (Table 1.2). Only one study reported on how the combined effect of diapause and acclimation affected

temperature tolerance. In a study on *T. variable*, Abdelghany *et al.* (2015) showed that the two factors were synergistic, with acclimation being the main factor that increases cold-tolerance in this insect.

Variation among studies confounds the detection of general patterns (Table 1.2). The larval stages appear to be the most cold-tolerant in the majority of dermestid species, particularly for *T. granarium* (Mansbridge, 1936, Solomon & Adamson, 1955). To achieve > 90% mortality for larvae held at −10^{o}C, 30 d is required for *T. granarium* (Mathlein, 1961), compared to 6 h for *T. inclusum* (Reguzzi *et al.*, 2011).

Low temperatures are more likely than high temperatures to be used in thermal treatments for pest control because, although longer exposure is needed to reach mortality, they are less likely to damage the treated product (Evans, 1986, Linnie, 1999). On products with low moisture content, temperatures as low as −29^{o}C have little effect on product quality but, in most products, too-high temperatures can lead to deleterious chemical and biochemical changes and most of these depend on the way the heat is delivered (Evans, 1986). Avoiding damage to the product requires studies specific to the product to determine the combination of temperature and duration exposure needed to achieve control, followed by post-treatment studies to assess product quality (e.g., for processed foods, preserved specimens, textiles) or viability (e.g., for bulk seeds) (Burges & Burrell, 1964, Evans, 1986, Fields, 1992, Fields *et al.*, 2012).

4.2 High temperature

I identified 24 papers reporting on the control of dermestids with high temperatures (Table 1.3). The most recent of these was published in 2002 (Wright, 2002), only two of

the studies considered the effect of diapause, and no studies assessed the effect of acclimation on heat-tolerance. Diapause does not typically increase survival at temperatures used for heat treatments (50-60^{o}C), and for this reason the effect of diapause on heat tolerance has not been widely assessed (Evans, 1981). Wright (2002) reported that diapause does not increase heat tolerance in larvae of *T. variabile* held for 4 minutes at 56^{o}C. However, Battu *et al.* (1975) reported the need of a longer exposure time at 50^{o}C to reach 95% mortality in *T. granarium* larvae in diapause (20 h) compared to non-diapausing larvae (5.3 h).

The mechanisms of mortality are similar between extreme temperatures and desiccation, adaptations to dehydration may enhance temperature tolerance (Danks, 2000, Danks, 1996). Therefore, insects adapted to tolerate dry environments will likely be able to better tolerate extreme temperatures (Danks, 2000, Danks, 1996). *Trogoderma granarium* and *A. verbasci* appear to be the most heat-tolerant of the dermestids (Table 1.3). When held at 50^{o}C, exposure for more than 5 h is required to achieve > 90% mortality for these species; whereas a similar level of mortality can be achieved within minutes for other dermestid species. The result of *T. granarium* being more heat-tolerant is not unexpected given that it is endemic to hot and dry environments (Hinton, 1945, Banks, 1977, Eliopoulos, 2013). In contrast, *T. variable*, which has a Neartic origin (Table 1.1), seems to be less heat-tolerant, reaching > 90% mortality in less than 5 minutes at 50^{o}C (Kirkpatrick & Tilton, 1972, Wright, 2002). Studies on *A. flavipes* and *T. granarium* also suggest that relative humidity (R.H.) plays an important role in heat tolerance and when increased, it might increase heat tolerance (Lindgren *et al.*, 1955, Ayappa *et al.*, 1957).

Within the dermestids, *T. granarium* is among those that have greatest thermal tolerance such that temperature treatments sufficient for its control would likely suffice for other species (Howe, 1965, Fields, 1992). In general, the published literature indicates that dermestid species cannot survive exposures of more than 3 days at temperatures below −20^{o}C or more than 2 hours above 50-60^{o}C (Table 1.3). Future studies to control dermestids at high and low temperatures must include different life stages of the insect, a wide range of temperatures and exposure times, and standardization of the analytical methods to determine the lethal time for the 50 and 95% of the population (LT_{50} and LT_{95}). The most widely used regression for these purposes is probit regression, which allows calculation of the probability of an observation falling in any of the two categories of a binomial dependent variable (e.g. dead/not dead) (Robertson *et al.*, 2007). Probit regression must be used only when the mortality versus time data follows a sigmoidal shape and should be reported with its variance estimates (fiducial limits) in order to be comparable (Fields, 1992).

4.3 Models

Mathematical models can be used to design effective thermal treatments for pest control. Such models require data on the survival of a species at different combinations of temperature and duration of exposure (Fields, 1992, Abdelghany *et al.*, 2015). Such data are normally obtained in laboratory studies for the most cold-/heat-tolerant life stage, including stages in diapause (Fields, 1992, Abdelghany *et al.*, 2015). Mortality of insects under constant temperatures can be modelled using kinetic or empirical models; e.g., modified fundamental kinetic and modified complementary log-log transformation models (Jian *et al.*, 2010). Mortality can be modelled as a function of time required for

the facility to reach the lethal temperature, the duration required for exposure to that lethal temperature, and the maximum/minimum temperature required for mortality (Subramanyam *et al.*, 2011). Mathematical models have also been developed for the use of non-constant temperatures to control stored product pests; e.g., heat accumulation models and heat treatment zones models (Jian *et al.*, 2013). Application of existing models may be condition dependent. Hence, models may require validation for a given combination of species, temperature range, and site of application (e.g., building, container, and product) (Jian *et al.*, 2010).

Models also can be developed to predict the risk of future global spread and establishment for a given insect pest (Paini & Yemshanov, 2012). This can be done with species occupancy models by identifying the optimal temperature and humidity for the pest development, reproduction and survival, and then matching this information to geographic regions where the pest does not yet occur (Banks, 1977). Such models have been developed to assess the risk of *T. granarium* arriving in Australia using a first-order Markov chain model (Paini & Yemshanov, 2012). This model used information on *T. granarium*'s current distribution and the probability of its presence in countries where it has not yet been reported to determine the most likely source countries and ports of entry into Australia (Paini & Yemshanov, 2012). Previous studies indicate that *T. granarium* requires conditions of > 20^{o}C average temperature for at least four months in a year to establish and reach pest status (Hadaway, 1956, Howe & Lindgren, 1957). Based on this information, mathematical models predict that *T. granarium* is unable to establish anywhere in Canada, except possibly in heated storage facilities (Ameen, 2012). However, these models may need to be reassessed, given new information on the thermal

tolerance of cold-acclimated insects or insects in diapause, plus a general warming of temperatures in recent decades due to climate change (Estay *et al.*, 2009).

The development of protocols using extreme temperatures to control dermestids is hindered by a general lack of information on the thermal tolerance of acclimated individuals, and on individuals in diapause. Diapause research on dermestids can be difficult because of the high degree of ecological variation among species and the diverse physiological and environmental cues that trigger diapause induction (Table 1.1) (Bell, 1994, Zhantiev, 2009). *Trogoderma granarium*, which exhibits density-dependent diapause, may be a particularly good model to study the effects of diapause on thermal tolerance because diapause induction is independent of temperature and, therefore, is not confounded by temperature acclimation (Nair & Desai, 1972, Abdelghany *et al.*, 2015).

The diapause stage in *T. granarium* makes its control difficult and enhances its pest status. Diapause allows these insects to use an intermittent food supply and survive adverse conditions including exposure to low temperature or to insecticides. The factors and their magnitude necessary to induce and terminate diapause must be clearly identified and methods to better identify diapausing individuals must be developed. The clear identification of other factors inducing and terminating diapause may lead to the manipulation of environmental conditions to prevent induction of the tolerant diapausing stage and the success of control methods; e.g., the use of extreme temperatures (Koštál, 2006, Wright & Cartledge, 1994, Nair & Desai, 1972). Although it has not been greatly assessed, diapause together with acclimation can increase significantly the temperature tolerance of dermestids (Fields, 1992, Abdelghany *et al.*, 2015). Studies on the development of strategies and models to control dermestid species (e.g., *T. granarium*)

should include the study of diapause and acclimated larvae. Larvae under these conditions are likely the most resistant to the method of control (Burges, 1962a, Wright & Cartledge, 1994, Denlinger, 2008). Studies on extreme temperatures needed for control of dermestid pests should also consider the species and life stages to be exposed, the rates of cooling and heating, environmental conditions used in the experiment, details of mathematical analyses used, and the consideration of the mortality at various temperatures to be used in mathematical modelling of the control method.

5. The insect microbiome and its effects on the host

Insects' microbial communities (microbiomes) mediate important biological and ecological traits that, when beneficial, facilitate insect adaptation to a changing environment (McFall-Ngai *et al.*, 2013, Moran & Sloan, 2015). This relationship among insects living in close association with their microbiome is termed symbiosis, where microorganisms can have a positive, negative, or no effect on their host (Douglas, 2007, Brownlie & Johnson, 2009, Moran & Sloan, 2015). Symbionts can affect host fitness, resistance to natural enemies, resistance to pesticides, thermal tolerance, and host reproduction (Zindel *et al.*, 2011). Depending upon the relationship with the host, symbionts can be classified as either essential/obligate or non-essential/facultative symbionts (Douglas, 2007).

Essential or obligate mutualistic symbionts usually colonize specialized cells in the host called mycetomes. Within the mycetomes, symbionts produce nutrients for the host, complement the host's metabolic properties, fix nitrogen, or suppress the actions of deleterious genes in the host's genome (Douglas, 2007, Zindel *et al.*, 2011, Wernegreen,

2012). For example, the aphid *Acyrthosiphon pisum* feeds on plant phloem sap that lacks some essential amino acids. This aphid relies on the bacterium *Buchnera aphidicola,* a maternally inherited symbiont, which synthetizes amino acids and lives exclusively in the aphids' mycetomes (Russell & Moran, 2006).

Non-essential or facultative symbionts are not required for insect survival, but they can affect the host biology and survival under certain conditions (Douglas, 2007, Sicard *et al.*, 2014). For example, bacteria from the genera *Wolbachia* are widespread symbionts of arthropods and filarial nematodes (Floate *et al.*, 2006). *Wolbachia* are vertically transmitted and can manipulate host reproduction (Floate *et al.*, 2006). The relationship of *Wolbachia* with its hosts can range from mutualistic to parasitic, and in most host species, removal of these bacteria does not cause mortality (Werren *et al.*, 2008). These bacteria can affect reproduction by causing cytoplasmic incompatibility, parthenogenesis or feminization in the host's progeny (Floate *et al.*, 2006, Li *et al.*, 2014). In some cases, *Wolbachia* infection can supress the action of deleterious genes (Starr & Cline, 2002) or protect the host against pathogens (Hedges *et al.*, 2008). In other cases, it causes mortality and reduces the fecundity of the host (Min & Benzer, 1997, Floate *et al.*, 2006).

The nature of symbiotic relationships is highly complex and is affected by biotic and abiotic factors (Wernegreen, 2012). Key factors include temperature, host diet, host species, physiological condition, genotype, and interactions with other members of the microbiome (genotype-by-genotype-by-environment interaction) (Douglas, 2007, Zilber-Rosenberg & Rosenberg, 2008, Moran & Sloan, 2015). In the association of *A. pisum* described above, environmental conditions and at least nine transovarially-transmitted genomes are involved in the aphid physiology; i.e., nuclear, mitochondrial, essential

symbiont, secondary symbionts, and phages of the secondary symbionts (Montlor *et al.*, 2002, Sakurai *et al.*, 2005). In addition to the essential symbiont *B. aphidicola*, some aphids also harbour at least other three secondary symbiotic bacteria that are not essential, but affect the host's biology (Russell & Moran, 2006). The bacteria *Serratia symbiotica* and *Hamiltonella defensa* protect their aphid host from successful parasitism by parasitic wasps (Oliver *et al.*, 2005, Guay *et al.*, 2009). These bacteria also increase aphid fecundity and survival under high temperatures by protecting the essential symbiont (rescuing effects) (Montlor *et al.*, 2002, Guay *et al.*, 2009). In contrast, within a certain temperature range the secondary symbiont *Regiella insecticolla* enables the aphid to feed on white clover plants and protects the aphid from fungal pathogens, but decreases host survival under higher temperatures (Montlor *et al.*, 2002, Russell & Moran, 2006).

Despite the importance of symbiotic microorganisms on the host's biology and ecology, they are one of the most variable and underestimated factors affecting control programs (Sinkins *et al.*, 1997, Zindel *et al.*, 2011). Consideration of these symbionts may therefore provide new insights into the development or improvement of methods of control against the host (Sinkins *et al.*, 1997, Zindel *et al.*, 2011). Symbiotic relationships with a potential use for insect pest control must have one of the two following traits: insect dependence on its microorganisms (essential symbionts), or the capacity of microorganisms to alter insect traits important to their pest status (facultative symbionts) (Douglas, 2007). An insect dependent on its essential symbiont is susceptible to its removal; e.g., when microorganisms provide essential molecules to the host, the case of blood-feeding, xylem-feeding (e.g., the aphids-*Buchnera* relationship), phloem-feeding and some grain-feeding insects (Gosalbes *et al.*, 2010). The manipulation of the insect's phenotype can

also be done by manipulating its microbiome (facultative symbionts) and in this way affect its pest status (Douglas, 2007). Manipulation of microorganisms causes changes in insect phenotypes; e.g., their host range, ability to transmit pathogens, resistance to parasitism or to pathogens, and resistance to insecticides and extreme environments (Douglas, 2007). The microbiome can decrease the vector capacity of insect hosts that transmit human parasites and viruses, by activating the insects' defenses (immune priming) (Meister *et al.*, 2009, Engel & Moran, 2013). In *Anopheles* spp. mosquitos, the clearance of gut microbiota with antibiotics increases infections of the human parasite *Plasmodium* (Meister *et al.*, 2009). The mosquito re-infection with the gut microbiota inhibits the development of the parasite (Pumpuni *et al.*, 1993).

Symbiont-based methods of control have been developed to supress populations of insect pests or diminish the transmission of pathogens by insect vectors. Paratransgenesis aims to diminish the vector capacity of insects by modifying their symbiotic bacteria to express anti-parasite/anti-viral compounds (Beard *et al.*, 2002, Panagiotis & Bourtzis, 2007). The introduction of symbionts known to diminish populations of insect vectors or their capacity to transmit pathogens in human populations has also been used. *Wolbachia* infections in mosquitos can block pathogen transmission and reduce the host population by sterilizing males (Incompatible Insect Technique) (Panagiotis & Bourtzis, 2007, Bourtzis *et al.*, 2014). The mosquito *Aedes aegypti* transmits dengue, chikunguya, zika and yellow fever viruses as well as the malaria causing parasite, *Plasmodium* (Moreira *et al.*, 2009). *Aedes aegypti* is not naturally infected with *Wolbachia,* but when these bacteria are artificially introduced they block the infection, replication, and dissemination of these viruses and the *Plasmodium* parasite into new human hosts (Moreira *et al.*,

2009). This effect persists in *Wolbachia*-infected (with one or more strains of *Wolbachia*) mosquitos after their release and establishment in wild populations (Frentiu *et al.*, 2014, Joubert *et al.*, 2016). Also, the cytoplasmic incompatibility effect of *Wolbachia* allows the transmission of the *Wolbachia*-infection into the offspring (Frentiu *et al.*, 2014, Joubert *et al.*, 2016). These results reinforced the potential use of *Wolbachia*-based technology for biocontrol of these viral pathogens (Frentiu *et al.*, 2014, Joubert *et al.*, 2016). A worldwide initiative has been started to develop this method of control and establish *Wolbachia* in wild populations of *A. aegypti* in areas where these diseases are endemic.

Symbionts can also alter the host's resistance against entomopathogen invasion, by affecting nutrient competition, niche occupation, or immune priming (Engel & Moran, 2013). For example, in the locust *Schistocerca gregaria*, an increased diversity of its gut's microbial community was negatively correlated to the colonization success of the pathogenic bacterium *Serratia marcenses* (Dillon *et al.*, 2005). In contrast, when the gut microbial community of the gypsy moth larvae was removed, the insecticidal activity of the biocontrol agent *Bacillus thuringensis* was also abolished. The insecticidal activity was recovered with the re-introduction of some members of the microbial community (Broderick *et al.*, 2006). These effects of the microbiome have been used to develop insect probiotics to diminish the colonization of pathogens in beneficial insects; e.g., in bees (Engel & Moran, 2013).

Insect-microbiome interactions can alter the susceptibility of the host to insecticides (Engel & Moran, 2013). The alydid bug, *Riptortus pedestris* is a pest of leguminous crops that acquires the gut bacterial symbiont *Burkholderia* from the environment. These bacteria degrade fenitrothion, which causes the failure of this insecticide as a pest-control

method (Kikuchi *et al.*, 2012). In contrast, infections of *Rickettsia* symbionts in the whitefly *Bemisia tabaci* increase host susceptibility to insecticides (Kontsedalov *et al.*, 2008).

The examples presented above provide evidence that the development of a successful method of control for insect pests should also consider symbiont-insect interactions, their multitrophic effects, and the factors that affect these interactions (Thomas & Blanford, 2003, Zindel *et al.*, 2011). Obligate symbiotic bacteria are susceptible to extreme temperatures and might restrain the insect thermal tolerance, whereas some facultative symbionts might provide protection against thermal stress (Feldhaar & Gross, 2009, Wernegreen, 2012). In biocontrol programs, the host-symbiont interaction has been shown to be highly affected by temperature and, in contrast, the host tolerance to temperatures can be affected by its symbionts (Thomas & Blanford, 2003, Wernegreen, 2012).

5.1 Effect of temperature on microbiome-insect relationship

Temperature is important for the infection success, density, transmission, and activity of insect endosymbionts (Thomas & Blanford, 2003). Insects that rely on obligate/essential symbionts are the most vulnerable to extreme temperatures (Wernegreen, 2012). Many obligate symbionts are found exclusively inside their insect host (obligate intracellular bacteria) and show signs of co-evolution with their hosts (Feldhaar & Gross, 2009). This obligate-symbiont lifestyle has been accompanied by a remarkable reduction in the size of their genome, and the loss of genes that are no longer needed in the host cell's environment; e.g. degradative metabolic pathways, defense mechanisms, DNA repair

mechanisms, regulatory functions, and horizontal gene transfer (Feldhaar & Gross, 2009). The reduction of the symbiont's genome leads to a higher accumulation of unfavourable mutations that generate inefficiency of protein folding, reduction on protein stability, and reduced ability to repair the protein structure after damage caused by high temperatures (Feldhaar & Gross, 2009, Wernegreen, 2012). Because of the diminished capacity to repair their DNA and proteins, symbiotic bacteria have a narrow temperature tolerance range (Wernegreen, 2012). High temperatures have been reported to interfere in insect-microbe endosymbiosis and to result in the loss of the symbiont (Wernegreen, 2012). Examples of susceptibility to heat have been reported in the *Buchnera* essential symbiont of aphids (see example above) (Russell & Moran, 2006), *Rickettsia* symbionts in bed bugs (Chang, 1974), gut symbionts in stink bugs (Prado *et al.*, 2010), *Sitophilus oryzae* principal endosymbiont (SOPE) in *Sitophilus* grain weevils (Heddi *et al.*, 1999), obligate endosymbionts in *Rhizopertha dominica* (Buchner, 1965), *Spiroplasma* (Anbutsu *et al.*, 2008), and *Wolbachia* in multiple insect species (Van Opijnen & Breeuwer, 1999, Mouton *et al.*, 2007, Bordenstein & Bordenstein, 2011). For example, bacteria from the genus *Spiroplasma* cause female-biased sex ratios of their host insects by causing selective death of male offspring during embryogenesis (male-killing phenotype) (Anbutsu *et al.*, 2008). Infection of *Spiroplasma* in *Drosophila* is affected by low (Montenegro & Klaczko, 2004) and high temperatures (Malogolowkin, 1959). The male-killing phenotype is consistently observed through successive host generations at 25^{o}C (Anbutsu *et al.*, 2008). At temperatures lower than 18^{o}C the infection density and vertical transmission of *Spiroplasma* was supressed, whereas temperatures higher than 28^{o}C affected only its vertical transmission (Anbutsu *et al.*, 2008). Similarly, in *Oryzaephilus*

surinamensis, the obligate symbionts present in the insect's mycetomes can be eliminated by exposing the insect to low or high temperatures (Buchner, 1965).

5.2 Effect of microbiome on insect survival at extreme temperatures

Although rarely considered, facultative symbionts can alter host tolerance to extreme temperatures (Wernegreen, 2012). For example, whiteflies (*Bemisia tabaci*) containing *Rickettsia* as a secondary symbiont have a significantly higher tolerance to heat shock than individuals lacking *Rickettsia* (Brumin *et al.*, 2011). The presence of *Rickettsia* at normal rearing temperatures induces the expression of host genes required for thermotolerance; under high temperatures this indirectly leads to a higher tolerance (Brumin *et al.*, 2011). In the tick, *Ixodes scapularis*, cold tolerance at −20°C is due to the expression of an antifreeze glycoprotein induced by its bacterial endosymbiont, *Anaplasma phagocytophillum* (Neelakanta, 2010). In contrast, ice-nucleating bacteria can increase the supercooling point of insects to make them more susceptible to freezing and reduce their cold-tolerance (Dillon & Dillon, 2004). The bacteria *Pseudomonas syringae* expresses an ice-nucleating protein (INA) in its membrane, which promotes ice crystal formation (freezing) of supercooled water (Cochet & Widehem, 2000). The application of this bacterium on grain reduces the time necessary to kill stored-product insect pests with cold temperatures by increasing the insect's supercooling point (Fields, 1993).

5.3 The microbiome of *Trogoderma granarium*

Genomic and metagenomic analyses have confirmed that the nutritional role of insect-symbiotic bacteria associations is common in insects (Gosalbes *et al.*, 2010). These associations mainly occur in insects with diets that are unbalanced or difficult to digest;

e.g., phloem or xylem sap (low in proteins and lipids), blood (deficient in B vitamins), woody or keratinous materials (hard to digest, low nitrogen), and grain (Douglas, 2007, Fukatsu *et al.*, 2007, Gosalbes *et al.*, 2010, Zindel *et al.*, 2011). Diets based on grain and keratinous materials are common to dermestid beetles (Fukatsu *et al.*, 2007, Zhantiev, 2009). Grain-based diets provide the insects with starch and carbohydrates, but are deficient in other nutrients and essential vitamins (e.g., vitamin-B complex) (Gosalbes *et al.*, 2010). Several stored-product insects establish symbiotic relationships with microorganisms (mainly bacteria and yeasts) to supply this diet deficiency (see Table 1.4) (Gosalbes *et al.*, 2010, Shen & Dowd, 1991, Anand & Pant, 1978).

Keratinous materials, such as feathers and hairs, are difficult to digest for most animals and have a low nutritional content, therefore it is possible that insects that feed on them harbour symbiotic microorganisms (Fukatsu *et al.*, 2007). Keratin is a molecule formed by crosslinked proteins that makes it resistant to biodeterioration (resistant to solubilisation, proteolysis, and digestion) (Trivedi *et al.*, 1991, Fukatsu *et al.*, 2007). Enzymes able to degrade keratin have been isolated only from bacteria and fungi; keratin digestion in insects is yet not fully understood (Hughes & Vogler, 2006). Keratin also has a low nutritional content with an amino acid composition of mostly cysteine, but poor in other amino acids and vitamins. For these reasons, insects with diets based on keratin might harbour symbiotic microorganisms that either help with digestion and (or) supplement the insect diet, but this aspect has not been widely assessed (Fukatsu *et al.*, 2007). The bacterial population within the intestine of clothes moth larvae, *Tineola bisselliella*, was too small to play a significant role in the digestion of keratin (Crewther & McQuade, 1955). However, this study was done with classical microbiological

techniques that do not account for the uncultivable bacteria (Amman *et al.*, 1995, Feldhaar & Gross, 2009). Molecular studies on the chewing louse, *Columbicola columbae,* have identified a *Sodalis*-allied bacterial endosymbiont that might be involved in keratin digestion or in supplementing the insect's diet (Fukatsu *et al.*, 2007).

The decomposition of keratin is restricted to some groups of arthropods including dermestids, but information on the microbiome of these species is limited. Among the dermestids, the main genera feeding on keratin are *Dermestes* spp., *Attagenus* spp., *Anthrenus* spp., and some species of *Trogoderma* (Rajendran & Hajira Parveen, 2005, Fukatsu *et al.*, 2007, Eliopoulos, 2013). Studies using optical and scanning electron microscopy of the alimentary system of *Anthrenus flavipes* larvae showed a large number of bacterial cells and amoeba-like protozoans, which were found colonizing the wool-fibre fragments surface and interfibrillar spaces (Trivedi *et al.*, 1991). The authors concluded that these organisms may contribute by supplementing the additional nutritional requirements not available in wool fibres and by helping to digest keratin (Trivedi *et al.*, 1991). The bacteria associated with the digestive tract of dermestids have been reported in only three studies in *Dermestes* and *Anthrenus* spp. (Table 1.4), but there are no studies using molecular techniques (Shrewsbury & Barson, 1953, Garg, 1977, Trivedi *et al.*, 1991). The few studies available only tested for infections of bacteria that affect reproduction but do not assess their effects on dermestid hosts.

Bacteria that influence host reproduction (e.g., *Wolbachia, Cardinium, Rickettsia, Spiroplasma*) are widespread in arthropods, including many stored-product insect pests (Table 1.4) (Li *et al.*, 2015). Screening for these bacteria using PCR-specific primers for each bacterial genus can detect infected individuals (Floate *et al.*, 2006). Three dermestid

species have been reported to be infected with *Wolbachia*: *Attagenus unicolor*, *Anthrenus verbasci* and *Dermestes lardarius* (Table 1.4). Conversely, populations of *T. variabile* and *T. granarium* have tested negative for *Wolbachia* (Table 1.4) (Zchori-Fein & Perlman, 2004, Kageyama *et al.*, 2010, Li *et al.*, 2015). For the species of dermestids infected with *Wolbachia*, there is no information on its effects (Table 1.4). Also, there is no information on whether dermestids are infected with other bacteria known to affect host reproduction; e.g., *Cardinium, Rickettsia, Spiroplasma*.

The diet and tolerance of *T. granarium* might be due to its microbiome, but there are no studies assessing this factor or its potential to develop methods of control for this pest. The development of *T. granarium* requires essential amino acids and cholesterol in the diet (Bhattacharya & Pant, 1969, Agarwal, 1970). *Trogoderma granarium* has diet requirements that are similar to *Tribolium* spp., which do not harbour endosymbionts in mycetomes. For example, *T. granarium* cannot synthesize cholesterol from known cholesterol precursors and need sterol in the diet (Agarwal, 1970). Studies show that extracts of *T. granarium* tissue contain amino acids including alanine, proline, glycine, glutamic acid, aspartic acid, and tyrosine even when these amino acids are absent in the diet, which suggest they are synthesized by the insect or acquired from other unknown source (Bhattacharya & Pant, 1969). However, the authors this research did not assess the role of bacterial associates in the synthesis of these compounds. Information on the microbiome might lead to the improvement of methods of control and to assess if the microbiome is involved in the high tolerance of this pest to extreme conditions (Zindel *et al.*, 2011).

5.4 Tools to assess microbiome effects on insect hosts

Classical studies on insect microbiomes led to the discovery of microorganisms as a factor that greatly affects insects' biology, but overlooked the uncultivable bacteria that cannot be assessed by traditional methods (Buchner, 1965). These traditional methods include culture, isolation and microscopy of microorganisms. Classical culture methods are limited to the study of only 1% of the existing microorganisms, because the other 99% cannot be isolated and maintained in pure culture or in cell-free media (Amman *et al.*, 1995, Feldhaar & Gross, 2009). Microscopy methods allow the study of microorganisms that are easily detected free-living in the insect or in mycetomes (Buchner, 1965). Applying these two techniques, the first symbionts studied in insects were the ones with a role in insect nutrition (Buchner, 1965). This was done by comparing the fitness of insects with essential symbionts to that of insects of the same species that had been treated with antibiotics to remove these symbionts (Buchner, 1965). Other studies applied a method known as the *Tribolium* test. This test compares the performance of the studied insect to the performance of *Tribolium* (known for not having any obligate symbionts) on the same diet (Buchner, 1965). However, the taxonomic analysis of the bacteria living in symbiosis with insects was difficult (Buchner, 1965, Feldhaar & Gross, 2009).

The development of molecular methods built upon classical methods by allowing the sequencing and identification of symbiotic bacteria, but also overlooked some members of the microbiome (Feldhaar & Gross, 2009). The diversity of an insect's microbiome can be determined using traditional molecular methods that include gene-targeting PCR, fluorescent *in situ* hybridization (Neelakanta, 2010), and molecular fingerprinting techniques (e.g., denaturing gradient gel electrophoresis-DGGE) (Nelson, 2008, Shi *et al.*,

2010). However, many of these techniques are laborious, require previous knowledge on the symbiont, and do not allow for a comprehensive analysis of the microbial community (Nelson, 2008, Shi *et al.*, 2010). These techniques are most useful when the targeted bacterium has already been identified and sequenced, or to only assess the diversity of the ecosystem (Shi *et al.*, 2010).

The development of Sanger sequencing improved the characterization of insect microbiome by allowing the sequencing of 16S rRNA genes of bacteria (Sanger *et al.*, 1977). This technology is a combination of many methods, which makes it expensive and laborious (Chial, 2008, Metzker, 2010). However, using the Sanger method the DNA sequence of a specific symbiont was determined and identified by comparing the symbiont DNA-sequence to known sequences of other bacteria (Feldhaar & Gross, 2009). Using this method, it was determined that most of the obligate symbionts are members of the Class gamma-Proteobacteria, Bacteroidetes, and beta-Proteobacteria into the family Enterobacteriaceae (Feldhaar & Gross, 2009). Sanger sequencing can be used to sequence whole genomes, but it has a high cost and is a laborious process (Metzker, 2010). Thus, the method had limited uptake for the widespread sequencing of whole genomes, or in metagenomics studies to characterize the microbiome of an organism (Metzker, 2010, Shi *et al.*, 2010).

The development of next-generation sequencing (NGS) technologies has overcome some of the limitations of the Sanger method (Margulies *et al.*, 2005, Shendure *et al.*, 2005, Metzker, 2010). NGS technologies are high-throughput methods, which make them more economical, less time-consuming, and more informative (Metzker, 2010). NGS methods are able to sequence high numbers of sequences cheaply and in a short time by

multiplexing, a strategy whereby a single volume of reagent amplifies and sequence millions of DNA molecules simultaneously in a single instrument run (Margulies *et al.*, 2005, Shendure *et al.*, 2005). For example, the sequencing of the first human genome by Sanger sequencing took 13 years with the help of multiple laboratories around the world and at a cost of $10 - 25 million (Margulies *et al.*, 2005, Chial, 2008). In contrast, NGS methods can sequence human genomes in less than a week for a few thousand dollars (Marusina, 2012). NGS technologies keep evolving by decreasing their error rates and cost, and are now being developed for a wide range of applications; e.g., RNA sequencing, metagenomics (Metzker, 2010). The main challenge in the evolution of NGS technologies is the analysis of the billions of sequences that are produced (Metzker, 2010). There is a need to improve the existing infrastructure of the information technology systems and bioinformatics programs to keep pace with the continuing developments in NGS technologies (Metzker, 2010).

The development of NGS technologies has facilitated the study of obligate symbionts, as well as the whole microbial community associated with an insect (Feldhaar & Gross, 2009, Shi *et al.*, 2010, McCutcheon & Moran, 2012). The sequencing of the whole genome of known symbionts allows predictions of their biological functions in the host (Feldhaar & Gross, 2009). Examples of symbionts whose genomes have been sequenced include those for symbionts of aphids (*Buchnera* spp.), carpenter ants (*Blochmania* spp.), psyllids (*Carsonella ruddii*), tsetse flies (*Wigglesworthia glossinidia*), of *Blattabacterium* from cockroaches and termites, and of *Candidatus* Sulcia muelleri in the maize leafhopper (Feldhaar & Gross, 2009, McCutcheon & Moran, 2012).

Metagenomics can be used to study the composition and dynamics of insect microbiomes (Nelson, 2008, Shi *et al.*, 2010). Metagenomics or community genomics is defined as the study of the metagenome, the totality of the genetic material of the microbial community existing in a determined ecological environment (Nelson, 2008, Shi *et al.*, 2010). The metagenomics approach allows a global view of the composition, diversity, and function of the microbial community including the uncultivable microorganisms (Shi *et al.*, 2010). It also provides additional information to understand the interactions involving communication, competition, collaboration, and resource partitioning of symbiotic microbial communities (Russell & Moran, 2006, Shi *et al.*, 2010). Interactions among microorganisms can be highly important for host biology in addition to the effects of each species of bacteria independently (Russell & Moran, 2006).

Metagenome sequencing is only the first step towards understanding the composition, dynamics and functions of the insect microbiome (Shi *et al.*, 2010). Sequencing itself doesn't allow for an understanding of protein activity and dynamic functional changes of the system. However, it does survey the diversity of the community and identify important features that facilitate more complex studies (Nelson, 2008). The study can be further extended to study the functional genes coding for specific enzymes in the community (functional metagenomics), the analysis of RNA of a microbial community to discover novel genes and their dynamics (metatranscriptomics), and the study of all the proteins in a microbial community (metaproteomics) (Nelson, 2008, Shi *et al.*, 2010). The microbiome of dermestids has not been studied from a genomic approach, but applying metagenomics sequencing of *T. granarium* will characterize the diversity and composition of its microbiome. This would be the first step to understand the microbiome

effects on the biology of *T. granarium*, which could be applied for future development and improvement of pest-control strategies.

STRUCTURE OF THE THESIS AND GOALS OF EACH CHAPTER

Chapter 2 Control of *Trogoderma granarium* with low temperatures

This chapter describes four experiments to assess the cold-tolerance of *T. granarium* and the combination of time and temperature necessary to control it. In the first experiment, the effect of density of larvae on the percentage of diapause was assessed to obtain larvae in diapause for the three following experiments. The second experiment was done to indirectly assess the cold-tolerance of *T. granarium* by measuring the supercooling points of the different life stages. The third experiment was done to determine the most cold-tolerant life stage. This was done by exposing all life stages at -10^{o}C for different durations to calculate the lethal times for 50 and 95% of the population (LT_{50} and LT_{95}). The fourth experiment used the most cold-tolerant life stage (diapausing-acclimated larvae) to determine its LT_{50}, $LT_{95,}$ LT_{99} and Probit 9 at four low temperatures, and assessed the termination of diapause after exposure to low temperatures.

Chapter 3 Control of *Trogoderma granarium* with high temperatures

This chapter describes two experiments investigating the combination of time and high temperature necessary to control *T. granarium*. In the first experiment, the most heat-tolerant life stage was determined by exposure of all the life stages to 45^{o}C for different exposure times. The lethal times for each life stage were determined. In the second experiment, the most heat-tolerant life stage (identified in Experiment 1 as diapausing

larvae) was exposed to five different high temperatures and the lethal times for 50 and 95% (LT_{50} and LT_{95}) of the population were determined.

Chapter 4 The microbiome of *Trogoderma granarium*

This chapter uses next-generation sequencing of the 16S rRNA gene to determine the effects of temperature and life stage on the composition of the bacteria associated with *T. granarium*. This chapter includes three experiments. The first experiment evaluated interspecific differences in the bacterial community associated with adults of *T. granarium* and its close relative, *T. variabile*. The second experiment described the bacteria associated with different life stages (eggs, larvae, diapausing larvae, acclimated-diapausing larvae, pupae, and adults). The third experiment evaluated the effect of temperatures on the bacteria associated with *T. granarium* adults.

Chapter 5 Discussion and conclusions

Chapter 5 summarizes the overall findings and general conclusions. The results of Chapters 2 to 4 are discussed and synthesized. The contribution of the thesis results for the control of *T. granarium*, and recommendations and future approaches also are discussed.

Table 1.1 Biology and life history of the most economically important species of dermestids.

Species	Common name	Geographic distribution	Tempe-rature (°C)[a]	R.H. (%)[a]	Larval stages (#)	Developmental time (days)	Adult longevity (days)	Diapause	Ecology	References
Anthrenus coloratus (Reitter)	Asian carpet beetle	Southeast Europe, England, USA, Africa, Asia	33-35	70-80	6-9	115 -120 (univoltine)	-	Yes	Stored product and museum specimen pest	Ali (1997)
Anthrenus flavipes (LeConte)	Furniture carpet beetle	Nearly cosmopolitan	35	90	18-20	246-248 (bivoltine)	-	Yes (obligate)	Animal products, museum pests	Griswold & Greenwald (1941); Ayappa *et al.* (1957)
*Anthrenus sarnicus (*Mroczkowski)	Guernsey carpet beetle	Europe	25	70	8	337	28	Yes (obligate)	Pest of dried animal collections in museums	Coombs & Woodroffe (1983); Armes (1988)
Anthrenus verbasci (Linnaeus)	Varied carpet beetle	Cosmopolitan	20	70	3-5	192	27	Yes (obligate)	Pest of dried insect collections/silk worms pupae	Griswold & Greenwald (1941); Blake (1958)
Anthrenocerus australis (Hope)	Australian carpet beetle	Europe and Australia	25	60-70	5-6	246	30	Yes (obligate)	Pest of dried dairy products, wool and other fabrics	Gerard & Ruf (1997)
Attagenus augustatus (Ballion)	-	China, Russia, middle east and India	29	-	-	730 (biennial)	150- 300	-	Pest of textiles, woolen fabrics and grains	Veer & Rao (1995)
Attagenus cyphonoides (Reitter)	-	Nearctic, Oriental and Palaearctic regions	22-27	45-70	-	118-132 (univoltine)	10-14	Yes (obligate), larvae overwinter	Woolen and other animal products	Veer *et al.* (1991)

Species	Common name	Geographic distribution	Tempe-rature (°C)[a]	R.H. (%)[a]	Larval stages (#)	Developmental time (days)	Adult longevity (days)	Diapause	Ecology	References
Attagenus elongatulus (Casey)	"yellow" black carpet beetle	Palaearctic region	27	50-70	-	90-95 days	18-20	Yes (crowded)	Infests plant and animal materials: woolens, felt and processed foods	Barak & Burkholder (1977)
Attagenus indicus (Kalik)	-	India, Nepal	27	75	-	240-270 days (univoltine)	144-229	Hibernates as larvae or adult	Stored woolen fabrics and carpets	Veer & Rao (1995)
Attagenus unicolor (Brahm)	Black carpet beetle	Cosmopolitan	28	65	5-11	> 326	3-35	Yes (obligate and larval crowding)	Pest of dead animal and vegetal materials	Back & Cotton (1938); Griswold & Greenwald (1941); Baker (1982), Baker (1977)
Attagenus fasciatus (Thunberg)	Banded black carpet beetle	Europe, nearly cosmopolitan	35	80	8-14	136 (bivoltine)	26-30	-	Attacks animal and vegetal products in storage	Ali *et al.* (2011)
Attagenus lobatus (Rosenhauer)	-	North Africa, South Europe, USA, Asia	25-27	65-70	-	253-287 (univoltine)	78-112	Overwinters as larvae or pupae	Woolen products	Veer *et al.* (1991)
Attagenus scalaris (Pic)	Black carpet beetle	Egypt, Libya	30	50	8-22	90-385	17-28	-	Stored textiles of animal origin	Abdel-Rahman *et al.* (1981)
Attagenus smirnovi (Zhantiev)	Brown carpet beetle	Europe, Africa	20	65	-	22-32	20	Yes	Animal and vegetal museum collections	Hansen *et al.* (2012)
Dermestes ater (Degeer)	Black larder beetle	Cosmopolitan	25	40-80	-	64-77	100-200	-	Pest on raw animal products	Coombs (1981)

Species	Common name	Geographic distribution	Tempe-rature (°C)[a]	R.H. (%)[a]	Larval stages (#)	Developmental time (days)	Adult longevity (days)	Diapause	Ecology	References
Dermestes haemorrhoidalis (Küster)	Black larder beetle, sheepskin dermestid	Nearly cosmopolitan	25	65	-	95-112	110-194	-	Animal and vegetal products, insect collections	Coombs (1979)
Dermestes lardarius (Linnaeus)	Larder beetle	Cosmopolitan	25	65	7	48-50	244	-	Scavengers/animal products	Coombs (1978); Jacob & Fleming (1984)
Dermestes maculatus (DeGeer)	Leather beetle	Cosmopolitan	33	-	6-8	34-40	14	Yes (crowded)	Stored produced of animal and vegetal origin	Osuji (1975); Cloud & Collison (1986)
Dermestes peruvianus (LaPorte de Castelnau)	Peruvian larder beetle	South America, USA, Europe, Asia	25	65	-	65-73	33-192	-	Plant and animal material	Coombs (1979)
Trogoderma anthrenoides (Sharp)	-	America, Europe, Asia	35	70	-	41-46	7-10	Yes (isolation)	Pest of plant and animal stored-products and museum specimens	Burges & Burrell (1964)
Trogoderma variabile (Ballion)	Warehouse beetle	Europe, Nearctic	32	70	5-6	30-46	14	Yes (isolation)	Pest of stored grain	Loschiavo (1960); Partida & Strong (1975)
Trogoderma granarium (Everts)	Khapra beetle	Europe, Asia and Africa	35	73	4-7	24-36	12-14	Yes (crowded, isolation)	Major pest of animal and vegetal stored products (mainly grain and cereals)	Hinton (1945); Hadaway (1956); Burges (1962a)
Trogoderma versicolor (Creutzer)	European larger cabinet beetle	Palaearctic region	30	73	-	30-43	12-16	-	Pest of dried animal and vegetal products/insect collections	Hadaway (1956)

Species	Common name	Geographic distribution	Tempe-rature (^{o}C)[a]	R.H. (%)[a]	Larval stages (#)	Developmental time (days)	Adult longevity (days)	Diapause	Ecology	References
Trogoderma inclusum (LeConte)	Larger cabinet beetle	Nearly cosmopolitan	35	73	4-7	36-42	3-16	Yes (isolation)	Minor stored product pest	Strong (1975); Klein & Beck (1980)
Trogoderma angustum (Solier)	Cabinet beetle	America, introduced to Asia and Europe	-	-	-	-	-	Yes (isolation)	Textiles and insect collections	Bell (1994)
Trogoderma sternale (Jayne)	Cabinet beetle	Nearctic	-	-	-	-	-	Yes (isolation)	Minor stored product pest	Bell (1994)
Trogoderma glabrum (Herbst)	Glabrous carpet beetle	Holarctic region (temperate)	27-38	-	5-6	30-49	6-14	Yes (isolation)	Minor stored product pest	Beck (1971a); Strong (1975); Klein & Beck (1980)
Trogoderma ornatum (Say)	Ornate cabinet beetle	Neartic region	-	-	-	-	-	Yes	Minor stored product pest	Bell (1994)
Trogoderma simplex (Jayne)	Plain cabinet beetle	USA and Canada	32	50	5-6	63-77	10-22	Yes (isolation)	Minor stored product pest	Strong & Mead (1975)
Trogoderma grassmani (Beal)	Tiny cabinet beetle	USA and Canada	-	-	-	-	-	Yes (isolation)	Minor stored product pest	Bell (1994)

- Blank cells represent unavailable information
[a] Temperature and humidity for the reported developmental time (days)

Table 1.2. Duration of exposure to sub-zero temperatures required to induce mortality for different species of economically important dermestid beetles.

Species	Reference	Life stage	Accli-mation	Tempe-rature (°C)	Duration	Mor-tality (%)
Trogoderma granarium	Lindgren & Vincent (1959)	Starved larvae	-	−10 to −7	36 d	40
		Fed larvae	-	−10 to −7	36 d	100
	Mathlein (1961)	Larvae	Yes	−2	180 d	45
				−5	90 d	23
				−10	30 d	97
				−19	15 d	100
					10 d	100
	Solomon & Adamson (1955)	Larvae	-	−3 to −8	10 mo	<4
	Voelkel (1924) cited in Hinton (1945)	Larvae	Yes	−10	72 h	11
		Larvae	No	−10	25 h	73
		Larvae	No	−16	24 h	98
		Adults	No	−16	16 h	100
	Eliopoulos *et al.* (2011)	Young larvae	-	−16	4 h	100
		Old larvae		−16	24 h	95
		Young adult		−16	12 h	92
		old adult		−16	12 h	97

Species	Reference	Life stage	Accli-mation	Tempe-rature (°C)	Duration	Mor-tality (%)
	Zacher (1938) cited in Strang (1992)	Larvae 4th instar	-	−10	25 h	50
	Reynolds & Rundle (1967)	Larvae	No	−10	0 - 3.5 h	3
					7.5 - 41 h	76
					48 - 72 h	91
	Wilches *et al.* (2014)	Eggs, Pupae, Adults	No	−10	20 d	100
		Larvae	No	−10	7 d	45
		Diapausing larvae	No	−10	7 d	25
Trogoderma variabile	Abdelghany *et al.* (2015)	Eggs	No	0	4 d	95
		Larvae	No	0	32 d	95
		Larvae	Yes	0	240 d	95
		Diapausing larvae	No	0	53 d	95
		Diapausing larvae	Yes	0	>275 d	50
		Larvae	No	−5	17 d	100
				−10	10 d	100
				−15	6 d	100
				−20	1 d	100

Species	Reference	Life stage	Acclimation	Temperature (°C)	Duration	Mortality (%)
		Diapausing larvae	Yes	−5	175 d	100
				−10	100 d	100
				−15	49 d	100
				−20	25 d	100
		Pupae	No	0	25 d	95
		Adults	No	0	15 d	95
Trogoderma inclusum	Reguzzi *et al.* (2011)	All life stages	No	−22	3 h	100
		Larvae	Yes	−22	3 h	90
		Adults	No	−10	5 h	95
		Eggs, larvae, pupae	No	−10	6 h	100
Trogoderma angustum	Bergh *et al.* (2006)	Larvae	No	−20	3 d	95
Attagenus fasciatus	Ali (1997)	Pupae	-	−5	7 h	100
		Pupae		0	6 d	100
Attagenus pellio	Florian (1986)	Eggs	-	−18	4 h	100
Attagenus smirnovi	Bergh *et al.* (2006)	Larvae	No	−20	1 d	100
				−14	1 d	100

Species	Reference	Life stage	Acclimation	Temperature (°C)	Duration	Mortality (%)
				−12	1 d	98
Attagenus unicolor	Back & Cotton (1926) cited in Strang (1992)	Larvae	-	−4	198 d	100
	Salt (1936)	Larvae	-	−22	several min	100
		Pupae/adults		−24	several min	100
Attagenus woodroffei	Bergh *et al.* (2006)	Larvae	No	−20	1 d	100
Anthrenus verbasci	Linnie (1999)	All life stages	-	−20	3 h	100
		Eggs, adults		−10	3 h	100
		Larvae		−10	24 h	10
		Pupae		−10	24 h	80
	Arevad (1979)	Eggs/pupae/adults	-	−20	2 h	100
	Bergh *et al.* (2006)	Larvae	No	−20	1 d	93
Anthrenus museorum	Arevad (1974) cited in Strang (1992)	Larvae	-	−20	2h	<100
	Arevad (1979)	Adults	-	−20	1 h	100
		Eggs		−20	6 h	100
	Bergh *et al.* (2006)	Larvae	No	−20	3 d	70

Species	Reference	Life stage	Accli-mation	Tempe-rature (°C)	Duration	Mor-tality (%)
Anthrenus flavipes	Back & Cotton (1926) cited in Strang (1992)	All life stages	-	−18	1 d	100
Dermestes vorax	Dawson (1984) cited in Strang (1992)	Larvae	-	−15	6 d	100
Dermestes maculatus	Strang (1992)	All life stages	-	−23	6 h	100
	Linnie (1999)	Eggs	-	−10	6 h	100
		Larvae, pupae		−10	24 h	< 35
		Adults		−10	9 h	100
		All life stages		−20	3 h	100
	Zhang (2012)	Adults	No	−20	6 h	100
Reesa vespulae	Bergh *et al.* (2006)	Larvae	No	−20	3 d	100
		Larvae		−20	1 d	80
	Mehl (1975) cited in Strang (1992)	Larvae	-	−20	2 d	>90
		Larvae		−10	14 d	>90
	Arevad (1974) cited in Strang (1992)	Larvae	-	−20	1 h	>90

- Blank cells represent unavailable information

Table 1.3. Duration of exposure to high temperatures required to reach > 90% mortality for different species of economically important dermestid beetles; individuals were not acclimated prior to exposure.

Species	Reference	Life stage	R.H. (%)	Tempe-rature (°C)	Duration	Mortality (%)
Attagenus scalaris	Abdel-Rahman *et al.* (1981)	Larvae	-	50	35 min	100
Attagenus fasciatus	Ali (1997)	Pupae	-	45	18 h	100
				50	30 min	100
	Ali (1992)	Larvae	-	50	20 min	100
Attagenus pellio	Zacher (1927) cited in Strang (1992)	Eggs/ larvae	-	52	20 min	100
Anthrenus verbasci	Linnie (1999)	All life stages	-	50	6 h	100
Anthrenus flavipes	Ayappa *et al.* (1957)	Pupae	30	41	2 h	100
			90	41	1 h	100
Anthrenus coloratus	Ali (1997)	Larvae	-	50	12 min	100
Anthrenus sarnicus	Armes (1985) cited in Linnie (1999)	All life stages	-	40	2 h	>90
Dermestes maculatus	Nakayama *et al.* (1983)	All life stages	-	50	30-60 min	>90

Species	Reference	Life stage	R.H. (%)	Tempe-rature (°C)	Duration	Mortality (%)
	Linnie (1999)	All life stages	-	50	3 h	100
Dermestes coarctatus	Yokoyama (1927) cited in Strang (1992)	Larvae/pupae/adults	-	50	1 h	100
				55	15 min	100
Trogoderma granarium	Battu *et al.* (1975)	Diapausing larvae	70	42	16 d	95
				45	6 d	95
				50	20 h	95
		Larvae	70	42	13 d	95
				45	4.2 d	95
				50	5.3 h	95
	Husain & Bhasin (1921)	Larvae	-	50	5 h	>90
		Larvae		54	20 min	>90
				60	4 min	>90
	Lindgren & Vincent (1959)	Eggs	-	57	3 min	95
	Lindgren *et al.* (1955)	All life stages	75	55	8 min	95
			95	55	15min	95
	Fleurat Lessard (1985)	larvae	-	70	16 sec	100

Species	Reference	Life stage	R.H. (%)	Tempe-rature (°C)	Duration	Mortality (%)
	Ismail *et al.* (1988)	All life stages	-	60	30 min	100
	Taheri (1988)	All life stages	-	45	4 d	100
	Mookherjee *et al.* (1968)	Pupae	-	50	16 h	100
		Pupae	-	55	2h	100
	Saxena *et al.* (1992)	Pupae	-	45	48 h	84
				55	2 h	100
	Ahmadani (2009)	Larvae	-	60	2 min	100
		Larvae		57	5 min	100
		Larvae		54	12 h	100
		Larvae		51	48 h	100
	Zacher (1927) cited in Strang (1992)	Larvae	-	50	5.6 h	>90
				52	1.5 h	>90
				53	0.5 h	>90
				54	20 min	>90
				55	10 min	>90
				58	5 min	>90
				71	1 min	>90

Species	Reference	Life stage	R.H. (%)	Tempe-rature (°C)	Duration	Mortality (%)
				82	0.5 min	>90
	Wilches *et al.* (2014)	Eggs, Pupae, Adults	-	45	7 d	>90
	Wright (2002)	Eggs	0	56	4 min	98.6
		Small larvae			2 min	91
Trogoderma variabile		Large larvae			4 min	100
		Diapausing larvae			4 min	93.3
		Pupae			4 min	100
		Adult female			4 min	96.4
	Kirkpatrick & Tilton (1972)	Adults	-	40	>24 h	>90
				45	12 h	>90
				50	5 min	>90
				55	1 min	>90

- Blank cells represent unavailable information

Table 1.4. Stored-product pests (Coleoptera) with known symbiotic relationships.

Family	Species	Gut-associated symbionts	Nutrition-associated symbiont effects	Reproductive symbionts*	Reproductive symbionts effects	Reference
Anobiidae	*Lasioderma serricorne* (Fabricius)	Yeast-like symbionts in midgut caecae: *Symbiotaphrina kochii.*	Production of vitamins (i.e., vitamin of the B-complex), sterols, essential amino acids and substrate detoxification (tobacco).	*Wolbachia* (+/-) depending on the strain. *Cardinium* (-)	Unknown	Buchner (1965) Dowd (1989) Ashworth (1993) Zchori-Fein & Perlman (2004) Kageyama *et al.* (2010) Li *et al.* (2015)
	Stegobium paniceum (Linnaeus)	Yeast-like symbionts in mycetomes: *Symbiotaphrina buchneri* .	Production of vitamins (i.e., vitamin of the B-complex), sterols and essential amino acids.	*Wolbachia* (+/-) depending on the strain. *Cardinium* (-)	Unknown	Buchner (1965) Dowd (1989) Ashworth (1993) Zchori-Fein & Perlman (2004) Kageyama *et al.* (2010) Li *et al.* (2015)
Bostrichidae	*Prostephanus truncatus* (Horn)	Bacterial endosymbionts in mycetomes.	Involved in the host's catabolism of nutrients.	*Wolbachia* (-)	Unknown	Nansen & Meikle (2002) Li *et al.* (2015)

Family	Species	Gut-associated symbionts	Nutrition-associated symbiont effects	Reproductive symbionts*	Reproductive symbionts effects	Reference
	Rhyzopertha dominica (Fabricius)	*Aeromonas liquifaciens* (Pseudomonadales: Pseudomonadaceae) in mycetomes.	Supply the insect with essential vitamins, nitrogen, and degradation of cellulose.	*Wolbachia* (-) *Cardinium* (-)	Unknown	Buchner (1965) Edde (2012) Zchori-Fein & Perlman (2004) Kageyama *et al.* (2010) Li *et al.* (2015)
Curculionidae	*Sitophilus oryzae* (Linnaeus) *S. zeamais* (Motschulsky)	*S.oryzae* and *S. zeamais* principal endosymbiont (SOPE and SZPE): *Sodalis*-allied bacterium in mycetomes. (γ- proteobacteria: Enterobacteriaceae)	Production of vitamins; i.e., vitamin B. Influence the energy-dependent performances.	*Wolbachia* (+/-) depending on the strain, *Cardinium* (-)	Cytoplasmic incompatibility.	Buchner (1965) Heddi *et al.* (1999) Heddi & Nardon (2005) Zchori-Fein & Perlman (2004) Kageyama *et al.* (2010) Li *et al.* (2015)
Dermestidae	*Attagenus unicolor* (Brahm)	No known essential/obligated symbionts	NA	*Wolbachia (+)*	Unknown	Li *et al.* (2015)
	Anthrenus verbasci (Linnaeus)	No known essential/obligated symbionts	NA	*Wolbachia (+)*	Unknown	Kageyama *et al.* (2010)

Family	Species	Gut-associated symbionts	Nutrition-associated symbiont effects	Reproductive symbionts*	Reproductive symbionts effects	Reference
	Dermestes frischii (Kugelann)	No known essential/obligated symbionts. Presence of *E. coli*, *Staphylococcus* spp., *Proteus* spp. and other Enterobacteriaceae members**	Unknown	*Wolbachia* (-)	Unknown	Garg (1977)
	Dermestes lardarius (Linnaeus)	No known essential/obligated symbionts. Presence of *B. mycoides*, *Staphylococcus* spp., *Streptococcus* spp.**	Unknown	*Wolbachia* (+)	Unknown	Shrewsbury & Barson (1953)
	Trogoderma granarium (Everts)	No known essential/obligated symbionts	NA	*Wolbachia* (-) *Cardinium* (-)	Unknown	Zchori-Fein & Perlman (2004) Kageyama *et al.* (2010) Li *et al.* (2015)
	Trogoderma variabile (Ballion)	No known essential/obligated symbionts	NA	*Wolbachia (-)*	Unknown	Li *et al.* (2015)
Silvanidae	*Oryzaephilus surinamensis* (Linnaeus)	Filamentous bacteria in mycetomes.	Production of vitamins, i.e., vitamin B.	*Wolbachia* (+) *Cardinium* (-)	Unknown	Buchner (1965) Li *et al.* (2015)

Family	Species	Gut-associated symbionts	Nutrition-associated symbiont effects	Reproductive symbionts*	Reproductive symbionts effects	Reference
Tenebrionidae	*Tribolium castaneum* (Herbst)	No known essential/obligated symbionts	NA	*Wolbachia* (-) *Cardinium* (-) *Rickettsia* (+) *Spiroplasma* (+)	Unknown	Kageyama *et al.* (2010) Goodacre *et al.* (2015)
	Tribolium confusum (Jacquelin du Val)	No known essential/obligated symbionts	Supplementation with bacteria isolated from *T. confusum* allowed its survival on a diet on which it would be unable for the insect to survive.	*Wolbachia (+)* *Cardinium (-)* *Rickettsia* (-) *Spiroplasma* (-)	Wolbachia induces cytoplasmic incompatibility, decrease (females) or increase (males) of fertility.	Van Wyk *et al.* (1959) Kageyama *et al.* (2010) Goodacre *et al.* (2015) Li *et al.* (2015) Fields *et al.* (unpublished data)

*Screening with PCR using primers specific for genera of bacteria known to affect reproduction: *Wolbachia*, *Cardinium*, *Rickettsia* and *Sitophilus*. (+) infected, (-) uninfected, (+/-) infected or uninfected depending on the insect strain.
** Isolation using traditional microbiological techniques (cultures) and biochemical reactions for identification.

CHAPTER 2: CONTROL OF *Trogoderma granarium* WITH LOW TEMPERATURES

Abstract

The khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) is a pest of stored grain in Africa, Asia and Europe. It is a quarantine insect for much of the rest of the world, including Canada, United States, Australia, New Zealand, and South America. Control of *T. granarium* can be achieved with methyl bromide, but this fumigant is an ozone-depleting substance and is being phased out world-wide. Thus, there is an urgent need to find new methods of control including the use of low temperatures. The general objective of this chapter is to report the duration necessary to control *T. granarium* at low temperatures. First, the induction of diapause with high density was investigated. The percentage of larvae in diapause increased with higher density of larvae reaching 50% at 73 larvae/g diet. Therefore, a high density of larvae was used to obtain diapausing larvae for the following experiments. The cold tolerance of *T. granarium* was indirectly assessed by the supercooling points (SCP) of different life stages. Eggs had the lowest supercooling point (average −26.2, minimum −28.1°C to maximum −24.2°C) followed by diapausing-acclimated larvae (−21.5°C, −25.2 to −16.5°C) = pupae (−21.3°C , −24.8 to −16.9°C) = acclimated larvae (−19.9°C , −25.4 to−15.3°C) = diapausing larvae (−19.6°C, −24.6°C to −12.1°C) = adults (−18.7°C, −21.8 to −15.5°C) < non-diapausing non-acclimated larvae (−14.4°C ,−20.5 to −10.3°C). The most cold-tolerant life stage was identified by exposing each life stage to −10^{o}C (selected because of the high quantity of data at this temperature) for various durations. According to their LT_{50} (estimated time until 50% mortality), the most cold-tolerant stage was the diapausing-acclimated larvae

(87 d, CI = 78-97 d) followed by acclimated larvae (51 d, 39 - 85 d) > diapausing larvae (19 d, 17 - 21 d) > adults (3.6 d, 3.1 - 4.1 d) > non-diapausing non-acclimated larvae (2 d, 0.5 – 3 d) > pupae (0.4 d, 0.30 - 0.42 d) > eggs (0.2 d, 0.04 – 0.32 d). The times for 99.9968% (Probit 9) mortality of diapausing-acclimated larvae were 532, 437, 305, 70 d at 0, −5, −10, and −15°C, respectively. Because of the long exposure time needed to control *T. granarium* at −15°C, cooling to below the SCP of −25°C will give immediate control and may be the best way to control this insect with low temperature.

Introduction

Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), is an economically important pest of stored products that is widespread in Asia, Africa, and Europe due to international trade (Hagstrum & Subramanyam, 2009, Eliopoulos, 2013). This insect is a quarantine pest for Canada, United States, South America, Australia, and New Zealand where, in order to avoid the introduction of *T. granarium*, commodities are mainly treated with insecticides and fumigants (Hagstrum & Subramanyam, 2009, Eliopoulos, 2013). The importance of *T. granarium* as a pest is partially due to its larval diapause, which increases its tolerance to insecticides (e.g., phosphine), starvation, low temperature, high temperature, and desiccation (Lindgren & Vincent, 1959, Bell *et al.*, 1984). The most commonly used method of control for *T. granarium* is fumigation with methyl bromide (Fields & White, 2002). However, this fumigant is being de-registered because of the damage it causes to the environment as an ozone-depleting substance (Fields & White, 2002). Although fumigation for quarantine is still permitted under the Montreal Protocol, the European Union has banned it even for this purpose (MBTOC, 2014). For this reason, alternatives for the control of *T. granarium* are needed.

All stored-product insects tested to date are freeze-intolerant and avoid death at sub-zero temperatures by avoiding freezing. Freeze-intolerant insects remain in an unfrozen supercooled state below their equilibrium freezing point (melting point) until they finally freeze at their supercooling point (SCP) (Lee, 1991, Fields, 1992). To lower their SCP, insects use mechanisms that include the loss of water, elimination of ice nucleators that encourage ice formation (e.g., food particles), increase of cryoprotectants such as glycerol, and production of anti-freeze proteins (thermal hysteresis proteins) (Lee, 1991, Danks, 1996, Danks, 2006, Doucet *et al.*, 2009). The SCP of a population often has a unimodal distribution and its range represents the proportion of the population that is at higher risk of freezing death (Bale, 1987). The SCP of insects that infest stored-products or museum-collections ranges from −4 to −22^{o}C, but there is still a significant mortality at temperatures higher than the SCP (Evans, 1986, Strang, 1992, Fields *et al.*, 1998, Linnie, 1999, Fields, 2001, Fields *et al.*, 2012, Fields, 1992). The survival of insects at temperatures above their SCP is time and temperature dependent with shorter durations of exposure and warmer temperatures resulting in increased survival. Laboratory experiments exposing individuals at each temperature for different exposure times are designed to calculate the combination of temperature and duration needed to kill a given percentage of the population (Fields, 1992, Fields & White, 2002, Eliopoulos *et al.*, 2011). Of those that have been investigated, the most cold-tolerant stored-product insects are *Sitophilus granarius* (Coleoptera: Curculionidae) and *Trogoderma granarium* (Coleoptera: Dermestidae) (Fields, 1992).

The high cold-tolerance of *T. granarium* might be due to its facultative diapause, which enhances its tolerance to other extreme conditions (Burges, 1962a, Tauber *et al.*, 1986,

Pullin, 1996). Diapause is an endocrine-mediated dormancy characterized by a low respiration rate (Beck, 1971a) and a longer larval stage (Burges, 1959a, Burges, 1960, Burges, 1962a, Burges, 1962b, Nair & Desai, 1972, Nair & Desai, 1973a, Nair & Desai, 1973b). During this state, individuals can synchronize development within populations, and increase their tolerance to extreme temperatures, desiccation, and fumigants (Burges, 1962a, Tauber *et al.*, 1986, Pullin, 1996). In the facultative diapause of *T. granarium*, the mature larvae feed and molt occasionally. Because facultative diapause depends upon environmental conditions, a given generation of an insect may or may not enter diapause (Denlinger, 1991, Bell, 1994). Diapause in *T. granarium* is initiated and maintained by external cues including exposure to low temperatures, inadequate food, and rearing in isolated or crowded conditions (Burges, 1962a, Nair & Desai, 1972). The termination of diapause in this insect can be spontaneous or occur with changes in density of larvae, or a rapid increase in temperature or photoperiod (Loschiavo, 1960, Burges, 1961, Beck, 1971b, Beck, 1971a, Wright & Cartledge, 1994).

The relation between diapause and cold tolerance is not always clear; some elements of diapause and cold tolerance are linked whereas others are not (Danks, 2006, Danks, 2005). The co-occurrence of diapause and cold tolerance may be coincidental or reflect independent responses to similar inductive cues. In addition, cold tolerance may occur in the absence of diapause (and vice versa) and may increase with previous exposure to sub-zero low temperatures (acclimation) (Tauber *et al.*, 1986, Denlinger, 1991, Danks, 2006, Danks, 2005). The facultative diapause of *T. granarium* is well suited to study linkages among diapause, acclimation, and cold tolerance. This type of diapause can be triggered at optimal rearing temperatures by rearing in crowded conditions, which allows

differentiating the effects of diapause and acclimation on cold tolerance (Abdelghany *et al.*, 2015, Nair & Desai, 1972, Burges, 1963).

The development of protocols using low temperatures to control *T. granarium* has been hindered because of the great variation in methodology and results among studies, as well as the lack of information on the most cold-tolerant states (Wilches *et al.*, 2016). Studies on the cold tolerance of dermestids are difficult to access and do not consider the effect of diapause or acclimation, which are likely to increase cold-tolerance (Wilches *et al.*, 2016). Previous studies with *T. variabile* identified the diapausing-acclimated larvae as the most cold-tolerant life stage (Abdelghany *et al.*, 2015). The limited studies available suggest that diapause and acclimation also increase cold tolerance in *T. granarium.* Lindgren and Vincent (1959) demonstrated that starvation reduced the mortality of *T. granarium* larvae held at -10^{o}C (Table 1.2). These starving larvae were likely in diapause (Burges, 1960). Voelkel (1924) cited in Hinton (1945), reports an acclimation protocol before exposing the larvae of *T. granarium* to sub-zero temperatures and suggests that acclimation enhances survival at low temperatures. Results from these previous studies suggest that *T. granarium* is among the most cold-tolerant of stored-product insect pests and the temperature treatments sufficient for its control would likely suffice for other species (Howe, 1965, Fields, 1992).

Information on the mortality of *T. granarium*'s most cold-tolerant life stage under different temperature-time combinations is essential to develop mathematical models for the application of cold as method of control (Burges & Burrell, 1964, Strang, 1992, Fields, 2001). To estimate the time necessary to kill a given percentage of the population, the time-percent of mortality relationship at a given temperature is transformed from a

sigmoidal to a linear relationship (Fields, 1992). This transformation is often done with probit (normal distribution) or logit analysis (logistic distribution), and fiducial limits are obtained to estimate the lethal times of 50, 95, and 99% (LT_{50}, LT_{95}, LT_{99}) of the most cold-tolerant life stage, which are subsequently used to provide recommendations for pest control (Fields, 1992, Robertson *et al.*, 2007). For quarantine security uses, to avoid the introduction of exotic pests into a country, the United States of America and other countries require the estimation of the exposure times needed to kill 99.9968% (Probit 9) of the population (survival of 32/1,000,000) (Robertson *et al.*, 2007). The validity of this measure has been questioned, but is still an obligatory requirement to provide recommendations for the control of quarantine pests such as *T. granarium* (Robertson *et al.*, 2007).

In this chapter, I report results of four experiments that collectively identify the combination of time and low temperatures necessary to control *T. granarium*. Experiment 1 assessed the effect of larval density on diapause induction. The selected density conditions (38, 55 or 73 larvae/g diet) were used to obtain larvae in diapause for the three following experiments. Experiment 2 indirectly assessed the cold tolerance of *T. granarium* by measuring the supercooling points of different life stages. Experiment 3 directly determined the most cold-tolerant life stage by exposing all life stages at -10^{o}C for different durations; results were then used to calculate the LT_{50} and LT_{95} of each life stage. Experiment 4 used the most cold-tolerant life stage (diapausing-acclimated larvae) to determine its LT_{50}, LT_{95}, LT_{99} and Probit 9 at four low temperatures (0, −5, −10, -15^{o}C) and assessed the percentage of diapause termination after the cold treatments.

Methods

Study species and life stages tested

Individuals collected from stored wheat in a Pakistani grain market were used to first establish a colony of *T. granarium* in 2011 at the Grain Research Laboratory, Training and Storage Management Cell in the Department of Agricultural Entomology, University of Agriculture, Faisalabad, Pakistan. Larvae from this colony were used to start a second colony in 2013 at the Insect-Microbial Containment Facility (IMCF) at the Lethbridge Research and Development Center of Agriculture and Agri-Food Canada (AAFC-LRDC), Lethbridge, Alberta, Canada. The larvae were imported into Canada with permit number P-2013-01610 issued by the Canadian Food Inspection Agency. The culture was reared in 4-liter jars on 200 g of a mixture of ground dog food (Purina® Dog Chow®, Mississauga, Ontario, Canada), skim milk powder (Carnation® Milk, Markham, Ontario, Canada), wheat germ, and Brewer's yeast (MP Biomedicals, Santa Ana, California, United States) (ratio by weight of food media was: 47.5, 17.5, 17.5 and 17.5%, respectively) (Abdelghany *et al.*, 2015), and kept at 30^{o}C, 10-30% R.H. in 14 h light and 10 h dark conditions. All the experiments were done using the same diet mix that was used for rearing.

The life stages tested were eggs, pupae, unsexed adults, and larvae of four different states; i.e., non-diapausing non-acclimated larvae, acclimated larvae, diapausing larvae, diapausing-acclimated larvae. Eggs were removed with a fine-haired paint brush from Petri dishes in which 20 adults had been placed five days previously. The pupae and adult stages were obtained directly from the colony. Non-diapausing larvae were obtained by

rearing individuals at low densities (4 larvae/g) for 23 - 25 d after hatching, whereas diapausing larvae were obtained by rearing individuals at high densities (38, 55 or 73 larvae/g of diet) for 60 d after hatching (Nair & Desai, 1972). Larvae were acclimated by exposure to 15^{o}C for 14 d, then 10^{o}C for 14 d, and then held at 4^{o}C for 25d (Abdelghany *et al.*, 2015).

Experiment 1: Effect of density on diapause

The objective of this experiment was to find the relationship between larval density and diapause induction, and to select the rearing density required to obtain diapausing larvae for the subsequent experiments. Larvae (1-5 days post-eclosion) were obtained by placing 20 adults in a Petri-dish to allow them to mate and lay eggs. Hatching was monitored after 10 days and the larvae were collected with a fine paint brush. Different densities of recently hatched larvae were placed in glass Petri dishes (100 mm diameter, 15 mm height) with 6 g of diet mixed and reared at 30^{o}C. The densities evaluated were 1 (n = 30 dishes), 7 (n = 6), 25 (n = 6), 110 (n = 6), 230 (n = 6), 330 (n = 6), 440 (n = 8) larvae/6 g of diet, which corresponds to 1 larva/6 g, 1, 4, 18, 38, 55 and 73 larvae/g of diet. After 20 d, the Petri-dishes were monitored every three days to remove and count the pupae (i.e., a measure of non-diapausing larvae). In preliminary studies, larvae normally pupated after 30 - 35 d at 30^{o}C. Therefore, larvae that had not pupated after 45 days were considered to be in diapause (Burges, 1959a, Karnavar & Nair, 1969, Nair & Desai, 1972).

Experiment 2: Supercooling points

Supercooling points (SCP) were determined to indirectly assess the cold tolerance of different life stages; eggs (n = 34), pupae (n = 31), adults (n = 35), non-acclimated non-

diapausing larvae (n = 41), diapausing larvae (n = 42), acclimated larvae (n = 30), and diapausing-acclimated larvae (n = 36). To measure the SCP, individuals from each life stage were attached with petroleum jelly to a fine thermocouple (0.26 mm in diameter) inside a small vial, placed in a cooling thermostat (LAUDA-Brinkmann, LP ©. Delran, New Jersey, United States), and the temperature was dropped to −50°C by 1°C/min. Temperatures were recorded at one-second intervals with a 21X Micrologger (Campbell Scientific Inc. ©. Logan, Utah, United States). The data were graphed, and the sudden rise in temperature; i.e., heat of crystallization, was taken as the SCP.

Experiment 3: Determination of the most cold-tolerant life stage

The goal of this experiment was to confirm that the supercooling points found in Experiment 2 corresponded to the most cold-tolerant life stage, and to define the range of durations to be tested in Experiment 4. Individuals from each life stage (n = 20 per life stage, per duration) were placed in glass vials (2.8 cm diameter, 8 cm height) with 3 g of diet mix, and held at −10°C in a freezer (Scientemp™, Adrian, Michigan, United States) for different periods of time; i.e., eggs (0, 2, 4, 6, 18 and 20 h), pupae (0, 2, 4, 6, 12, 18, 24 h), adults (0, 0.5, 2, 4, 6, 7, 8 and 10 d), larvae (0, 2, 4, 6, 7, 8, 10 and 15 d), acclimated larvae (0, 5, 15, 30, 50 and 60 d), and diapausing-acclimated larvae (0, 30, 60, 90, 120, 150 and 240 d). These periods of time were selected according to the results from previous studies that examined the time required to achieve 0, 50 and 100% of mortality at −10°C (Table 1.2). After removal from −10°C, individuals were held at 30°C for five days. Mortality was subsequently assessed by observing eggs and pupae for eclosion, and larvae and adults for movement.

Experiment 4: Cold tolerance of diapausing-acclimated larvae at different low temperatures

The goal of this experiment was to assess the time necessary to kill 50 (LT_{50}), 95 (LT_{95}), 99 (LT_{99}) and 99.9968% (Probit 9) of the population of the most cold-tolerant life stage (diapausing-acclimated larvae) at 0, −5, −10, at −15°C. Diapausing larvae (20 per vial) were placed inside cylindrical glass vials (2.8 cm diameter, 8 cm height) with 3 g of media. The vials were then exposed to different time-temperature combinations (n = 3 vials per each time-temperature combination): 0°C (0, 60, 100, 160, and 200 d); −5°C (0, 60, 90, 120 and 190 d); −10°C (0, 30, 55, 90, 120, 190, 240 d) and −15°C (0, 15, 20, 25, 30, 35, and 50 d). After each exposure, the diapausing-acclimated larvae were moved to 30°C and the mortality was assessed after five days as described for larvae in Experiment 1. After 15 days, the termination of diapause was assessed as the percentage of larvae that survived the exposure and pupated (Burges, 1962b, Nair & Desai, 1973b).

Data analyses

To account for the mortality of larvae in each of the treatments in Experiment 1, the total number of surviving individuals in each sample was calculated as:

$$Total\ survivors = Number\ of\ pupae + Number\ of\ larvae\ after\ 45\ days$$

To determine if there was a significant effect of density on the proportion of diapause, a generalized linear model (GLM) with a quasibinomial distribution (i.e., logistic regression) was used. The quasibinomial distribution was chosen instead of the binomial distribution because the former considers the overdispersion observed in the data. The explanatory variables in the model were the density of larvae at the start of the

experiment (initial density) and the proportion of larval survival. The response variable was the proportion of diapause. The proportion of survival was included as an explanatory variable to ensure that the differences in the proportion of diapause were not due to differences in larval survival among density treatments. The proportion of diapause was evaluated over the number of individuals that survived per sample (Total survivors):

$$Proportion\ of\ diapause = Number\ of\ DL/Total\ survivors$$

The SCP data in Experiment 2 were not normally distributed for three of the seven life stages tested (Shapiro-Wilk test; $W = 0.920$ to 0.939, $P = 0.006$ to 0.047). Hence, data for all life stages were rank-transformed and then assessed for differences in SCP among life stages with a one-way ANOVA; differences among groups were analyzed with Tukey's HSD test. In Experiments 3 and 4, the exposure time necessary to kill 50 and 95% of the population (LT_{50} and LT_{95}) for each life stage or low temperature was calculated using probit analysis without logarithmic transformation of the exposure time. In Experiment 4, probit analysis was also used to calculate the time required for killing 99% (LT_{99}) and 99.9968% or probit 9. To determine the differences in the termination of diapause among times and temperature of exposure, the proportion of larvae terminating diapause was arcsine-square root transformed to approach normality and analyzed with a two-way ANOVA.

Statistical analyses were performed in PoloPlus 2.0, LeOra Software (Finney, 1971), R (Version 3.0.2) in Rstudio (Version 0.98.501- ©2009-2013 Rstudio, Inc.) and SYSTAT Software (Version 12.0, Systat Software, Inc.).

Results

Experiment 1: Effect of density on diapause

Results of the GLM did not detect an interaction between the proportion of survival and initial density of larvae ($F_{1, 58} = 0.03$; $P = 0.86$; $n = 68$), nor did it detect a significant main effect of proportion of survival on the proportion of diapause ($F_{1, 59} = 0.30$; $P = 0.58$; $n = 68$). The proportion of survival x density interaction and the proportion of survival were therefore removed from the model to create a simplified model. The simplified model included only initial density of larvae as an explanatory variable and proportion of diapause as a response variable. The simplified model generated with a quasibinomial distribution had an intercept of −1.98, slope of 0.005, and a parameter of overdispersion estimated to be 3158. This simplified GLM detected a significant effect of density of larvae on the proportion of diapause (Fig. 2.1; $F_{1, 60} = 56.00$; $P < 0.0001$; $n = 68$). According to the model, diapause increased at higher densities and reached an average above 25% at densities higher than 38 larvae/g (Fig. 2.1). Therefore, densities greater than 38 larvae/g were used to obtain diapausing larvae in the following experiments.

Experiment 2: Supercooling point (SCP)

The SCPs differed among life stages (Table 2.1; ANOVA; $F_{6, 242} = 68.85$; $P < 0.0001$; $n =$ 30 to 42 individuals); i.e., from coldest to warmest: eggs < diapausing-acclimated larvae = pupae = acclimated larvae = diapausing larvae = adults < non-diapausing non-acclimated larvae. These results indicate that eggs and diapausing-acclimated larvae are most cold-tolerant, whereas non-diapausing non-acclimated larvae and adults are least cold tolerant.

Among the larval life stages, acclimation and diapause affected the SCP. Acclimated stages had a significantly colder SCP than the non-acclimated ones; i.e., non-diapausing non-acclimated larvae < acclimated larvae (Tukey's HSD test; $P < 0.0001$), and diapausing larvae < diapausing-acclimated larvae (Tukey's HSD test; $P = 0.019$). The SCP of diapausing larvae was significantly colder than the SCP of non-acclimated non-diapausing larvae (Tukey's HSD test; $P < 0.0001$). No significant difference was detected in the SCP of diapausing-acclimated larvae and acclimated larvae (Tukey's HSD test; $P > 0.05$). The non-diapausing non-acclimated larvae had the warmest and the diapausing-acclimated larvae had the coldest mean SCP among larval stages.

The distribution of the SCPs for diapausing larvae, acclimated larvae and acclimated-diapausing larvae showed a slight bimodal nature (Fig. 2.2). The bimodality of the data was not statistically significant for diapausing larvae (Hartigans' dip test for unimodality; $D = 0.0434$, $P > 0.05$), acclimated larvae (Hartigans' dip test for unimodality; $D = 0.0474$, $P > 0.05$) or acclimated-diapausing larvae (Hartigans' dip test for unimodality; $D = 0.0586$, $P > 0.05$).

Experiment 3: Determination of the most cold-tolerant life stage

According to the time of exposure required at -10^{o}C for killing 50% of the individuals in each life stage (LT_{50}), the cold tolerance of each life stage from highest to lowest was diapausing-acclimated larvae > acclimated larvae > diapausing larvae > adults > non-diapausing non-acclimated larvae > pupae > eggs (Table 2.1). The X^2 goodness-of-fit statistic versus degrees of freedom for each life stage indicates that the probit model adequately fits the data (Table 2.1; $P > 0.05$).

The SCP did not consistently reflect cold tolerance. For example, eggs and pupae had some of the coldest SCPs, but died quickest at low temperatures (less cold tolerance). However, as suggested by their SCPs, diapause and acclimation were associated with increased cold tolerance of larval stages. Acclimation increases cold tolerance more than diapause; the mean LT_{50} for diapausing larvae (18.8 d) was 9 times longer than for non-diapausing non-acclimated larvae (2.1 d), and the mean LT_{50} for acclimated larvae (50.7 d) was 24 times longer than the LT_{50} for non-diapausing non-acclimated larvae (2.1 d). However, acclimation and diapause in combination increased cold tolerance more than when either condition was present separately. The mean LT_{50} for diapausing-acclimated larvae (87.2 d) was 4.6 times longer than the one for diapausing larvae (18.8 d) and 1.7 times longer than the mean LT_{50} for acclimated larvae (50.7 d). Diapausing-acclimated larvae were much more cold-tolerant (Table 2.1) compared with all life stages, ranging from 42times more cold-tolerant than non-diapausing non-acclimated larvae to 436 times more cold-tolerant when compared to eggs.

Experiment 4: Cold tolerance of diapausing-acclimated larvae at different low temperatures

The most cold-tolerant life stage, diapausing-acclimated larvae, was tested at different low temperatures. The mean LT_{50} for diapausing-acclimated larvae was 225 d at 0^{o}C, 147 d at -5^{o}C, 106 d at -10^{o}C and 25 d at -15^{o}C (Table 2.2). The time necessary to reach 99.9968% (probit 9) mortality was 532 d at 0^{o}C, 437 d at -5^{o}C, 305 d at -10^{o}C and 70 d at -15^{o}C (Table 2.2). The value of probit 9 is required for quarantine uses, but more replication is needed to obtain more precise estimates. The X^2 goodness-of-fit statistic

versus degrees of freedom for each temperature indicates that the probit model adequately fits the data (Table 2.2; $P < 0.05$).

There was lower termination of diapause with longer duration of exposure and lower temperatures (Fig. 2.3). The termination of diapause (percent larvae that pupated) was significantly different among temperatures (ANOVA; $F_{3, 56} = 37.2$; $P < 0.0001$; $n = 64$ larvae combined across time-treatment combinations), times of exposure (ANOVA; $F_{1, 56} = 10.18$; $P = 0.0023$; $n = 64$), and there was an interaction between both factors (ANOVA; $F_{3, 56} = 13.38$; $P < 0.0001$; $n = 64$). Almost 100% of the larvae that were not exposed to low temperatures (control) terminated diapause. Also more than 50% terminated diapause at exposure times shorter than 160 d at 0°C, 90 d at −5°C, 60 d at −10°C and 15 d at −15°C (Fig. 2.3).

Discussion

Diapause induction and termination

The mean incidence of larval diapause increased when larvae were reared at higher densities, reaching 61% at the highest density tested (Fig. 2.1). The high variability in the percentage of larvae that entered diapause (Fig. 2.1), also suggests that other factors different than density might be involved in triggering diapause (Burges, 1963, Nair & Desai, 1972). Burges (1963) reported the same trend in *T. granarium* at 30°C, reaching a maximum of about 50% at a density of 24 larvae/g of food. Nair and Desai (1972) reported a highly variable diapause induction of more than 85% versus 35%, for *T. granarium* larvae reared under crowded (33 larvae/g of diet) and isolated conditions (1 larva/g of diet), respectively. Differences in the levels of diapause among studies may

reflect several factors. Different populations or individuals of the same population of *T. granarium* may require different levels of stimuli to trigger diapause (Bowler & Terblanche, 2008). Genetic variation among populations may limit the potential for diapause induction (Nair & Desai, 1973a). Diet also may be a factor; e.g., Burges (1963) used wheat kernels and bran, whereas Nair and Desai (1972) used whole wheat flour as a source of food. Both of these diets are coarse in texture and have a low nutritional content (Athanassiou *et al.*, 2016). In contrast, the diet used in the present study was high in protein and was supplemented with yeast, a rich source of vitamin B that enhances the development of *T. granarium* (Bhattacharya & Pant, 1969).

The mechanism acting under crowded conditions to induce diapause in *T. granarium* is yet not fully understood. Early studies suggest induction reflects the presence of fecal pellets and, in particular, of linoleic acid (Burges, 1963, Ikan *et al.*, 1970, Nair & Desai, 1972). The stored-product pests *Plodia interpunctella* Hubner and *Ephestia cautella* Walker (Lepidoptera: Pyralidae) also have a diapause induced by crowding and low temperature (Hagstrum & Silhacek, 1980). In these insects, the mechanisms suggested to induce diapause are the presence of fecal pellets and mechanical contact between larvae (Nair & Desai, 1972, Hagstrum & Silhacek, 1980). Similar to *T. granarium*, in these insects diapause occurs even when no food is present at the time of crowding (Nair & Desai, 1972). Therefore, it is possible that mechanical contact among larvae also represents a mechanism of diapause induction in *T. granarium* (Nair & Desai, 1972). The intensity of this stimulus in addition to the number of fecal pellets would vary according to the degree of crowding, which could induce a higher proportion of diapause at higher densities of larvae (Nair & Desai, 1972). The induction of the density-dependent diapause

of *T. granarium* can be achieved at optimal high temperatures (30°C), which makes it favorable to study the separate effects of diapause and acclimation on cold tolerance (Abdelghany *et al.*, 2015).

After exposure to low temperatures, some of the *T. granarium* diapausing-acclimated larvae were able to complete development and terminate diapause. The percentage of termination of diapause (pupation) decreased with exposures for a longer time and at lower temperatures (Fig. 2.3). In previous studies of *T. granarium,* the removal of larvae from crowded conditions, provision of fresh food and increase in temperature terminated the density-dependent diapause (Burges, 1962b, Nair & Desai, 1973b). These observations agree with those of Experiment 4. Diapausing-acclimated larvae were kept in crowded conditions for 133 days, without providing fresh diet. However, the exposure to low temperatures was done using fresh diet, and at a lower density of larvae per sample (7 larvae/g of diet) than the density used for rearing (73 larvae/g of diet). The reason of the termination of diapause in the control (0 days exposure and no mortality; Figure 2.3) was likely caused by the lower density of larvae, and the provision of fresh food. For the other treatments, exposed to low temperatures, termination of diapause was likely caused by the combination of low density of larvae, fresh food, and the increase of the temperature up to 30^{o}C.

The initiation and termination of diapause in *T. granarium* can be induced without manipulating temperatures, but the termination of diapause did decrease in conditions of longer exposures at lower temperatures. In a previous study the biggest changes in temperature promoted pupation (Burges, 1962b), but this trend was not found in Experiment 4. The higher mortality and lower termination of diapause was achieved

under longer exposures and lower temperatures (Fig. 2.3). These results could be explained by the accumulation of more severe cold-related damage at longer times and colder temperatures (Denlinger & Lee 1998). The production of molecules common to cold tolerance and diapause (e.g., glycerol) can delay diapause termination (Denlinger & Lee 1998). These molecules also accumulate and reach higher concentrations at longer and colder exposures (Denlinger & Lee 1998).

There is intra-population variability in the diapause intensity of *T. granarium*, represented by variable durations of diapause under the same conditions (Burges, 1962b, Tauber & Tauber, 1976, Masaki, 2002). In other insects, diapause intensity is controlled by multiple genes and has high intra- and inter-population variability even at constant rearing conditions (Tauber & Tauber, 1976, Masaki, 2002). The high variability of the diapause intensity is commonly due to sex, geographic strain, and the influence of pre-diapause environmental conditions (Tauber & Tauber, 1976, Masaki, 2002). Studies in the behaviour of *T. granarium* in malt stores found that within an optimal temperature range, high percentages of diapausing larvae would leave crevices within the substrate and pupate (terminate diapause), whereas a smaller percentage would remain hidden in crevices. The latter group of larvae were considered to be most deeply in diapause and had not yet responded to the increase in temperature (Burges, 1962b).

The ability to undergo diapause as larvae in *T. granarium* evolved in their ancestral environment as a scavenger and was maintained in the stored-product environment (Beck, 1973, Armes, 1988, Armes, 1990). This state is beneficial to a stored-product pest, because it allows the insect to tolerate suboptimal conditions where the food supply is sporadic and unpredictable (Beck, 1973, Armes, 1988, Armes, 1990). Unlike most stored-

product pests, *T. granarium* does not fly, which limits its capacity to spread to other storage facilities with available food and potential mates (Burges, 1969). Diapause also allows individuals to synchronize development and to survive adverse conditions, including extreme temperatures and desiccation (Burges, 1962a, Tauber *et al.*, 1986, Pullin, 1996). The diapause stage terminates with optimal conditions for growth and reproduction. Adults of *T. granarium* that went through diapause are larger and lay more eggs, which would promote a rapid increase of the population after diapause (Karnavar, 1972). Future studies should identify triggers for induction and termination of diapause, study the effect of diapause and cold treatment on fecundity, develop methods to better identify diapausing individuals, and study the effect of deacclimation on cold tolerance.

Supercooling point (SCP) of different life stages and selection of the most cold-tolerant life stage

The results of Experiment 2 indicate that SCP is a stage-dependent variable in *T. granarium*. However, the SCP is not directly related to the cold tolerance of the different life stages when exposed at -10^{o}C (Experiment 2). The SCP results suggest that eggs, diapausing-acclimated larvae, and pupae would be the most cold tolerant life stages. However, eggs and pupae survived the shortest time when exposed at -10^{o}C (Table 2.1). This discrepancy can be explained by ontogenetic, physiological, and ecological differences among eggs, pupae and larval stages (Baust & Rojas, 1985, Denlinger & Lee 1998). Ice formation occurs at lower temperatures in eggs because they have a small volume of water and lack of ice-nucleating agents (Salt, 1961). Very few insects overwinter as eggs, reflecting their cold susceptibility (Fields, 1992). This cold susceptibility might be due to their low capacity to repair cold-related injury, cold

inactivation of proteins, or decoupling of the normal metabolic processes (Knight *et al.*, 1986, Lee, 1991, Mason & Strait, 1998). In other stored-product insect pests and on the close related *T. variabile*, eggs are also the least cold tolerant life stages (Fields, 1992, Abdelghany *et al.*, 2015). Because food particles can act as ice-nucleating agents in the digestive tract, non-feeding stages including pupae or eggs have lower SCP (Salt, 1961, Hammond, 2015). However, eggs and pupae cannot move into microenvironments protected from the extreme temperatures, which is a common mechanism used by larvae and adults to increase their cold-tolerance (Burges, 1959b, Bale, 1987, Hammond, 2015).

Supercooling points are useful as a first approach to the low cold-tolerance limits of organisms, in particular when studying the effects of acclimation and diapause (Bale, 1987). The SCPs are useful to define temperature limits and times to be used in mortality tests for different states of larvae. In my results, the SCPs were an accurate approach to the cold-tolerance of acclimated and diapausing larval stages. The distribution of the SCP provides the proportion of the population that is likely to die because of freezing under certain conditions (Bale, 1987). The bimodality of the SCPs distribution was non-significant and might be associated with the presence/absence of food in the larval gut (Fig. 2.2). The bimodal distribution of the SCP, although unusual, has been found in other insect populations and might be due to underlying mechanisms affecting cold-tolerance (e.g., ecological factors, sex, differences in acclimation, different amounts of ice-nucleators) (Block *et al.*, 1990, Renault *et al.*, 2002). The distribution can be further assessed by increasing the number of larvae tested and by controlling the food ingested by the larvae before the test.

The acclimated and diapausing larval stages had lower SCPs than their counterparts (Table 2.1). Diapause alone caused an 8-fold increase in the LT_{50} of non-diapausing non-acclimated larvae (Table 2.1). In the results by Lindgren and Vincent (1959), exposure of *T. granarium* larvae at −10°C for 36 days caused 40% mortality, whereas non-diapausing larvae had 100% mortality. Diapause and cold tolerance are related but not consistently linked. Phenomena that occur during diapause can increase cold tolerance only by coincidence rather than due to a causal link between both states; i.e., production of cryoprotectants, elimination of gut contents, and dehydration of body tissues (Lindgren & Vincent, 1959, Bell *et al.*, 1984, Tauber *et al.*, 1986, Danks, 2006, Danks, 1996, Danks, 2005). In addition, crowded conditions used to induce diapause can induce the production of proteins involved in cold tolerance (Sørensen *et al.*, 2003). Larval crowding in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) induces expression of heat-shock proteins, increases adult longevity, and increases resistance to thermal stress (Sørensen & Loeschcke, 2001).

Cold-related injuries are usually mitigated by previous exposure to less severe cold temperatures or acclimation (Denlinger & Lee 1998). Acclimation alone increased the LT_{50} by 23-fold compared to non-diapausing non-acclimated larvae (Table 2.1). During the acclimation period, the larvae can improve their capacity to supercool by the cessation of feeding, the excretion of ice-nucleators (food) from their digestive tract, the loss of water to reduce the chance of ice formation, or by the accumulation of cryoprotectants (e.g., glycerol) (Salt, 1961, Denlinger & Lee 1998). In insects, the SCP is reduced to around −20°C by the removal of ice-nucleating agents solely (Zachariassen, 1991). In addition, the concentration of glycerol increases 2 to 3-fold during acclimation, and

acclimation promotes the production of molecules that repair or reduce the cold-related damage; e.g., heat shock proteins (Lee, 1991, Denlinger & Lee 1998). In this way, the acclimated larvae are prepared for the upcoming colder temperatures. A previous study on *T. granarium* reported that larvae acclimated for 4 days had 98% mortality after 30 days at −10^{o}C (Mathlein, 1961). My results showed that 95 days were required to reach 95% mortality at −10^{o}C. The differences among these results are possibly due to the longer acclimation time (58 days) used in my experiments. In the congeneric *T. variabile*, non-diapausing larvae acclimated for 45 days had an LT_{50} 5 times greater than the LT_{50} for non-diapausing non-acclimated larvae (Abdelghany *et al.*, 2015).

Diapause increases cold-tolerance, and the combination of acclimation and diapause had a synergistic effect in *T. granarium*. Diapause induction by crowded conditions at high temperatures allowed separation of the effects due to diapause versus acclimation. Diapause alone and acclimation alone had an 8-fold and 23-fold increase in cold tolerance, respectively, whereas diapause and acclimation together had a 40.5-fold increase in cold tolerance when compared to non-diapausing non-acclimated larvae (Table 2.1). In *T. variabile*, there is also a synergistic effect of diapause and acclimation on cold tolerance (Abdelghany *et al.*, 2015). However, in *T. variabile*, cold tolerance increased only 14-fold with diapause and acclimation together, only 5-fold with acclimation alone and did not increase with diapause alone (Abdelghany *et al.*, 2015). Therefore, diapause can increase cold tolerance in *T. variable*, but only in combination with acclimation (Sørensen *et al.*, 2003, Abdelghany *et al.*, 2015). In *T. granarium*, the increase of cold-tolerance when diapause is present alone suggests that higher cold tolerance is a component of its diapause state; i.e., diapausing larvae are more cold-

tolerant than non-diapausing larvae despite the fact that both were reared at the same temperature (Abdelghany *et al.*, 2015). These results suggest that the initiation of diapause might be linked to enhanced cold hardiness, and a separate set of environmental cues are not needed to initiate the cold hardening process (Denlinger, 1991).

Cold tolerance of diapausing-acclimated larvae at different low temperatures

The design of control methods using cold temperatures for *T. granarium* must consider different temperatures and times for mortality of the most cold-tolerant life stage; i.e., the diapausing-acclimated larvae. Control could be achieved by decreasing the temperatures to the lowest SCP found for a diapausing-acclimated larvae (– 25.2^{o}C) (Table 2.1). However, higher temperatures for longer periods of time can also achieve mortality. According to my results, the diapausing-acclimated larvae can be controlled by exposures of 541, 340, 212 and 49 d at 0, −5, −10, −15^{o}C (value for the upper confidence interval of the LT_{95}), respectively (Table 2.2). The periods of time able to control the diapausing-acclimated larvae should suffice to control the other life stages of this pest and other less cold-tolerant insects including *T. variabile.* The diapausing-acclimated larvae is also the most cold-tolerant life stage of *T. variable*; at −10^{o}C it has an LT_{50} of 74 d (95% CI 50-184 d) (Table 1.2; Abdelghany *et al*., 2015) compared to 106 days (95% CI 97 – 115 d) for *T. granarium* (Table 2.2).

Trogoderma granarium is one of the most cold-tolerant stored-product pests despite its tropical origin (Eliopoulos, 2013). The LT_{50} for the most cold-tolerant life stages of the most cold-tolerant stored product insects exposed at 0^{o}C are 3 days for *Ephestia elutella*, 20 days for *Plodia interpuctella* and 3 days for *Sitophilus granarius,* whereas it is 221

days for *T. granarium* (Fields, 1992). Cold and desiccation cause dehydration and osmotic stress at a cellular level, and insects use similar mechanisms to respond to both stressors (Danks, 2000). At a molecular level, there is an overlap in the genes expressed during tolerance to both cold and desiccation (Sinclair *et al.*, 2013). Some of these genes are responsible for the accumulation of trehalose, which preserves the structure of proteins and membranes, and improves tolerance to cold, desiccation, and hypoxia (Sinclair *et al.*, 2013). These proteins also protect insects from damage related to other stresses and are produced when the insect is exposed to detrimental conditions such as extreme temperatures, UV radiation, heavy metals, pesticides, hypoxia, salinity, desiccation, high density, and bacterial or viral infection (Lindquist, 1986, Denlinger & Yocum, 1998, Fields, 2006, Fields, 1992, Sørensen *et al.*, 2003). Therefore, the cold-tolerance of *T. granarium* might be the result of cross-tolerance with desiccation; this insect could be pre-adapted to cold conditions due to its adaptations to dry and hot environments (Sinclair *et al.*, 2013).

The United States Department of Agriculture (USDA) requires reporting the 99.9968% (Probit 9) of mortality for treatments designed to avoid the introduction of exotic pests including *T. granarium* (Follett & Neven, 2006, Robertson *et al.*, 2007). To achieve Probit 9 mortality at the 95% confidence level, a minimum of 93,613 insects must be tested with no survivors (Follett & Neven, 2006). Although we did not test the required number of individuals to estimate Probit 9, an estimate of the time needed to reach this mortality at different temperatures was obtained. According to the results of Experiment 4, the Probit 9 upper confidence interval values calculated for *T. granarium* are 887, 592, 356 and 84 days at 0, −5, −10, −15^{o}C, respectively (Table 2.2). The wide confidence

interval obtained for the Probit 9 values calculated from the experimental data is not surprising, because the greatest differences are likely to appear at the extreme tails (close to 0 and 99%) of the mortality distributions (Robertson *et al.*, 2007). The estimated Probit 9 can be used in confirmatory tests by a specialized quarantine laboratory with the capacity to produce large numbers of individuals (Robertson *et al.*, 2007).

Conclusions

1. The induction of diapause in *T. granarium* increases by rearing at high densities of larvae even at optimal temperatures (30^{o}C), which makes *T. granarium* a model species for studying the separate effect of diapause and acclimation on cold tolerance.

2. Diapause and acclimation increase cold tolerance in *T. granarium* larvae. Although there is a greater effect of acclimation than diapause, there is a synergistic effect when both are present. Therefore, the diapausing-acclimated larvae are the most cold-tolerant life stage. Further research needs to be conducted to identify triggers for induction and termination of diapause, to clarify the effect of diapause and cold treatment on fecundity, to develop methods to better distinguish between diapausing and non-diapausing larvae, and to determine the effect of de-acclimation on cold tolerance.

3. *Trogoderma granarium* has a high cold tolerance, with a Probit 9 of 70 d at −15^{o}C for diapausing-acclimated larvae. The termination of diapause in diapausing-acclimated larvae after cold treatment depends on the temperature-time combination at which the larvae were exposed. Future studies should be done to develop temperature-time for mortality models to control *T. granarium*, assess incorporation of other methods to reduce

the period of low temperature exposure needed to achieve control, and to assess the effect of cold treatment on the quality of the treated product.

4. Due to the long durations needed to control *T. granarium*, reducing temperatures to below the SCP of −25^{o}C, which will give immediate control, may be the best way to control this insect with low temperature.

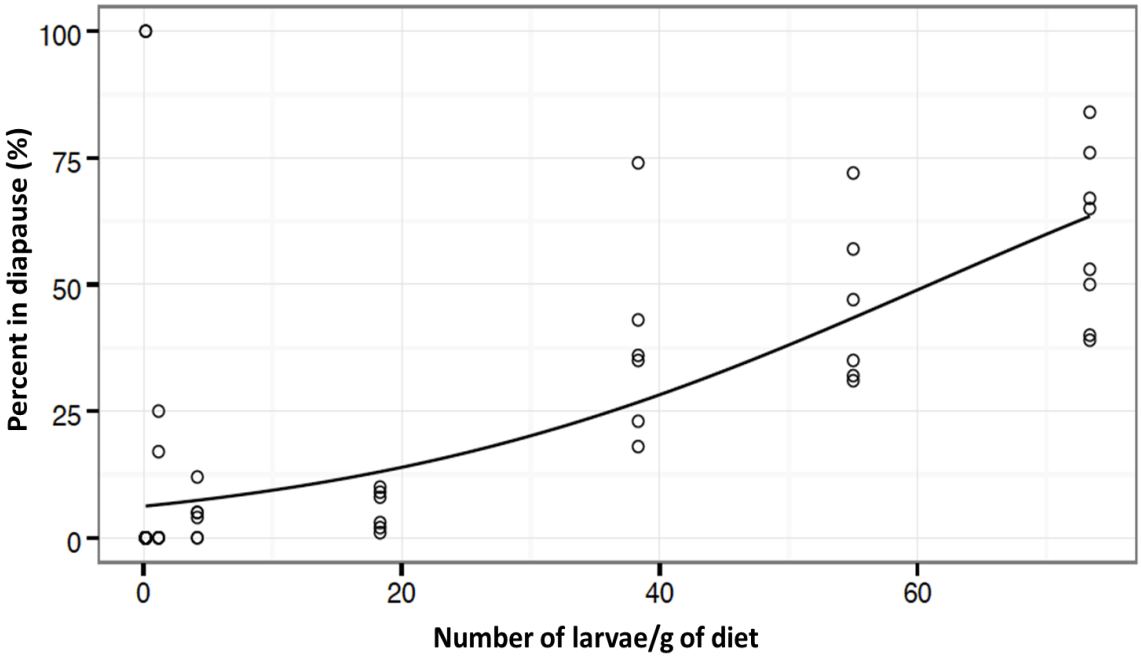


Figure 2.1. Logistic regression for the percent of larvae that undergo diapause when reared at different larval densities.

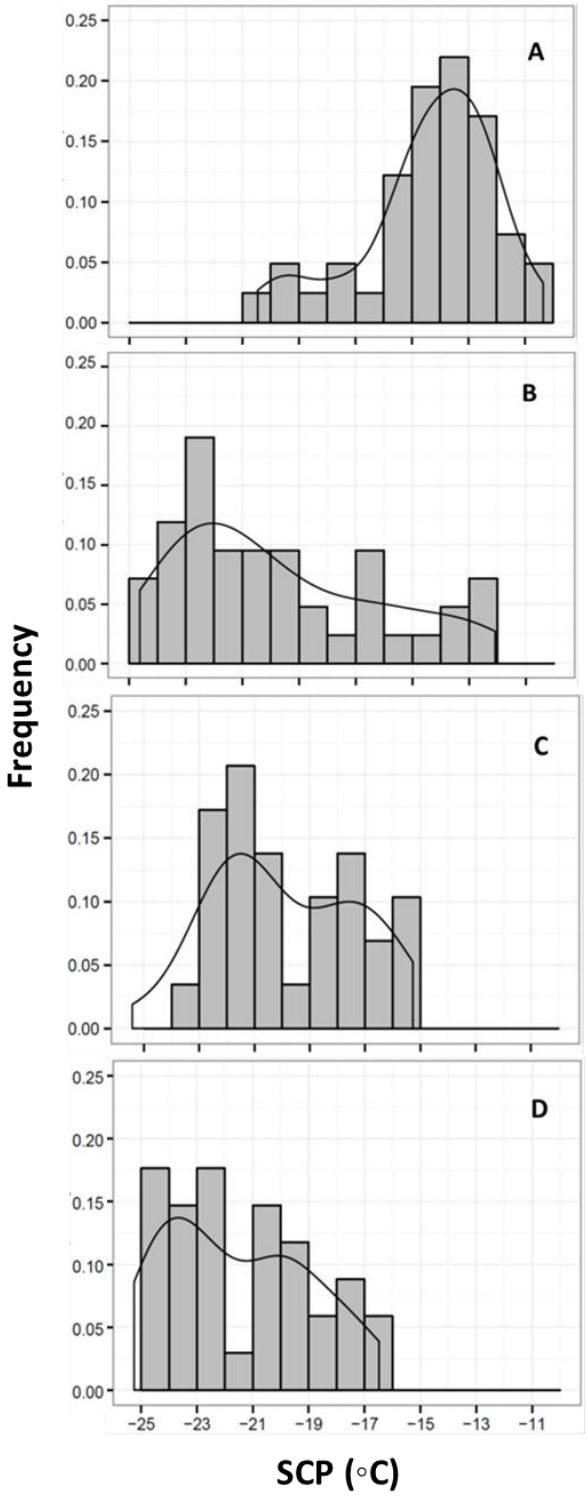


Figure 2.2 Frequency of Supercooling point (SCP) (^{o}C) values for different types of larvae. **A)** Non-diapausing, non-acclimated larvae (*n* = 41) **B)** Diapausing larvae (*n* = 42) **C)** Acclimated larvae (*n* = 30) **D)** Diapausing-acclimated larvae (*n* = 36).

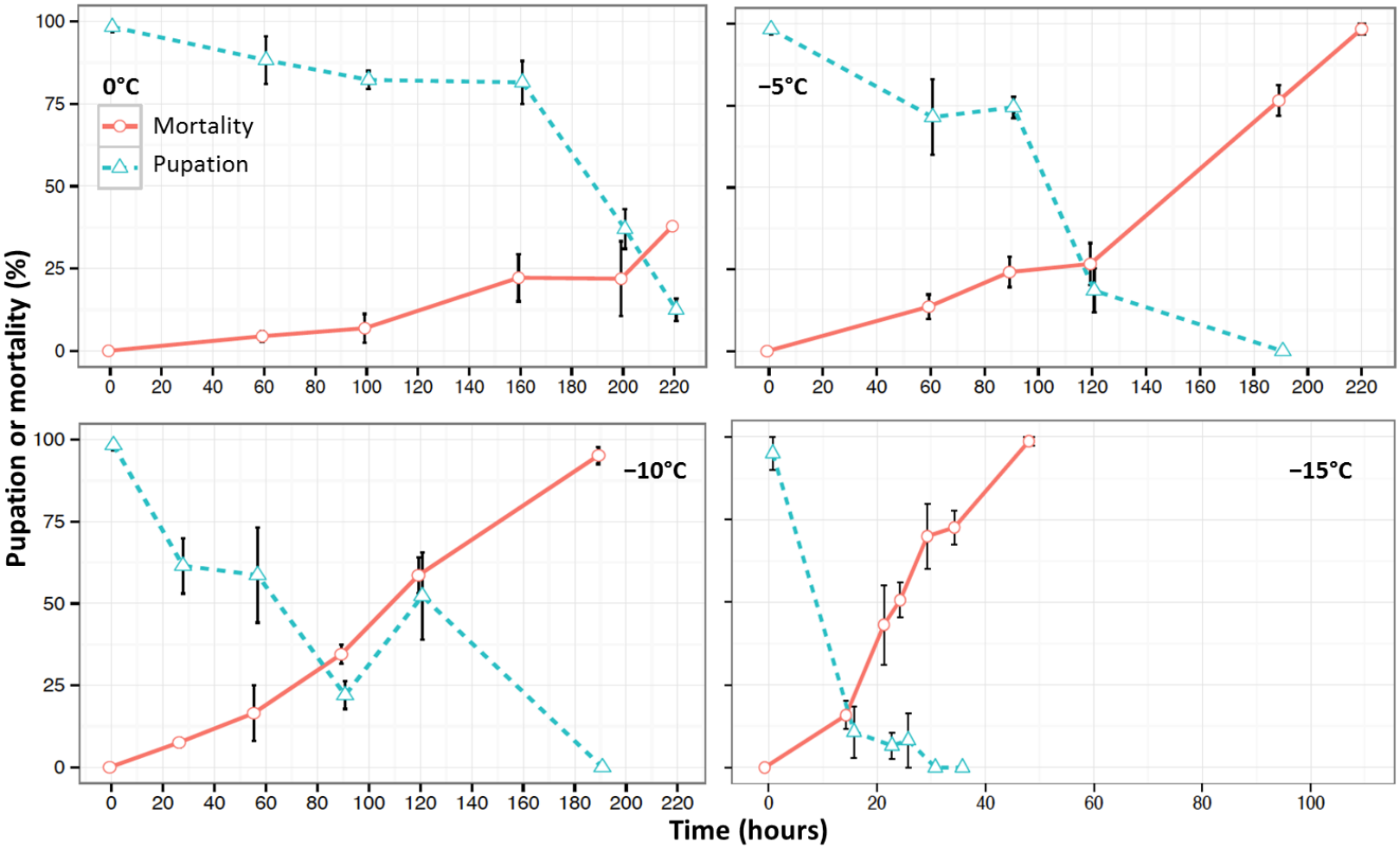


Figure 2.3 Effect of temperature and exposure time on mortality of diapausing-acclimated larvae and pupation of survivors 15 days after cold treatment (break of diapause). Each value is the mean (± SE) of three samples each containing 20 diapausing larvae at the onset of exposure.

Table 2.1. Experiment 2 and 3 - Supercooling point (SCP) and lethal time (LT) in days to kill 50 or 95% of the population of each life stage at −10°C.

Life stage	Accli-mation	Dia-pause	SCP ± SE* (max, min values)	LT_{50} (days) (95% CI)	LT_{95} (days) (95% CI)	Slope ± SE	Intercept ± SE	X^2 (df)
Eggs	No	No	−26.2 ± 0.1 ***e*** (−24.2, −28.1)	0.20 (0.04 - 0.32)	0.83 (0.65 – 1.19)	2.63 ± 0.32	−0.53 ± 0.14	50.4 (21)
Larvae	No	No	−14.4 ± 0.4 ***d*** (−10.3, −20.5)	2 (0.5 – 3)	11 (9.5 – 13)	0.19 ± 0.02	−0.39 ± 0.15	26.4 (25)
Larvae	Yes	No	−19.9 ± 0.5 ***abc*** (−15.3, −25.4)	51 (39 – 85)	95 (70 – 205)	0.037 ± 0.008	−1.866 ± 0.31	6.49 (5)
Larvae	No	Yes	−19.6 ± 0.6 ***bc*** (−12.1, −24.6)	19 (17 – 21)	37 (33 – 42)	0.092 ± 0.007	−1.73 ± 0.15	29.53 (22)
Larvae	Yes	Yes	−21.5 ± 0.4 ***a*** (−16.5, −25.2)	87 (78 – 97)	151 (136 – 176)	0.026 ± 0.003	−2.23 ± 0.32	9.82 (13)
Pupae	No	No	−21.3 ± 0.3 ***ab*** (−16.9, −24.8)	0.36 (0.30 – 0.42)	0.82 (0.72 – 0.96)	3.59 ± 0.27	−1.29 ± 0.12	47.44 (24)
Adults	No	No	−18.7 ± 0.3 ***c*** (−15.5, −21.8)	3.6 (3.1 – 4.1)	7 (6.3 – 8)	0.48 ± 0.03	−1.74 ± 0.15	46.88 (24)

*One-way ANOVA, $F = 68.85$; df = 6, 242; $P < 0.0001$, means followed by different letters are significantly different, Tukey's HSD test $P < 0.05$.

Table 2.2 Experiment 4 - LT_{50}, LT_{95}, LT_{99} and Probit 9 values for diapausing-acclimated larvae of *T. granarium* at different temperatures.

Temperature (± 1 °C)	**LT_{50} (days) (95% CI)**	**LT_{95} (days) (95% CI)**	**LT_{99} (days) (95% CI)**	**Probit 9 (days) (95% CI)**	**Slope ± SE**	**Intercept ± SE**	**X^2 (df)**
0	225 (193 – 303)	351 (282 – 541)	403 (318 – 641)	532 (406 – 887)	0.013 ± 0.003	-2.92 ± 0.42	9.27 (9)
−5	147 (131 – 170)	266 (227 – 340)	316 (265 – 413)	437 (357 – 592)	0.014 ± 0.002	-2.02 ± 0.22	16.8 (13)
−10	106 (97 – 115)	188 (171 – 212)	222 (200 - 253)	305 (270 - 356)	0.02 ± 0.002	-2.13 ± 0.22	10.49 (15)
−15	25 (23 – 27)	44 (40 – 49)	51 (46 - 59)	70 (62 - 84)	0.09 ± 0.009	-2.22 ± 0.24	28.27 (22)

CHAPTER 3: CONTROL OF *Trogoderma granarium* WITH HIGH TEMPERATURES

Abstract

Khapra beetle, *Trogoderma granarium* (Coleoptera: Dermestidae), is a pest of stored grain in Africa, Asia and Europe. It is a quarantine insect for much of the rest of the world and is often intercepted in food shipments being imported into the United States of America and Canada. Control of *T. granarium* can be achieved by fumigation with methyl bromide, but this fumigant is an ozone-depleting substance and is being phased out. Thus, there is an urgent need to find new methods of control including the use of high temperatures. In a two-step process, this Chapter investigates the exposure period required to control *T. granarium* at high temperatures. First, I determined the most heat-tolerant life stage. Second, and for a range of temperatures, I determined the period of exposure required to kill 50 and 95 percent of individuals in this life stage. In descending order, the most heat-tolerant life stages at 45^{o}C were: diapausing larvae (lethal time for 50% of the population (LT_{50}) = 5 d), non-diapausing larvae (LT_{50} = 2 d) > adults (LT_{50}= 1.4 d) > pupae (LT_{50}= 1 d) > eggs (LT_{50}= 0.4 d). The exposure time necessary to control (Probit 9) diapausing larvae was 397 h at 45^{o}C, 7 h at 50^{o}C, 1.2 h at 55^{o}C, and 1.2 h at 60^{o}C. An exposure of 1.2 - 2 h at 60^{o}C is recommended to control *T. granarium* with high temperatures.

Introduction

Khapra beetle, *Trogoderma granarium* (Coleoptera: Dermestidae) is an economically important pest of stored products that is widespread in Asia, Africa, and Europe due to

international trade (Hagstrum & Subramanyam, 2009, Eliopoulos, 2013). This insect is a quarantine pest for Canada, the United States, South America, Australia, and New Zealand, where efforts are in place to prevent its introduction (Hagstrum & Subramanyam, 2009, Eliopoulos, 2013). Fumigation with methyl bromide is widely used to control *T. granarium*, but this method is being de-registered because of its non-target effects to the environment and to human health (Fields & White, 2002). Although fumigation for quarantine purposes is still permitted under the Montreal Protocol, the European Union has banned it even for this purpose (MBTOC, 2014). For this reason, alternatives for the control of *T. granarium* are needed.

Exposing insects to high temperatures can be an environmentally safe method of pest control (Denlinger & Yocum, 1998, Bergh *et al.*, 2006). High temperatures affect cell-membrane fluidity, the rate of metabolic reactions, and the structure of macromolecules that are essential to maintain cell structure and function (i.e., RNA, DNA, proteins, lipids) (Denlinger & Yocum, 1998). Optimal temperatures for insects generally range between 25 - 35^{o}C (Fields, 1992, Strang, 1992). Suboptimal high temperatures (35 - 45^{o}C) are the maximum temperatures for reproduction of most species. Lethal temperatures (above 45^{o}C) cause the cessation of movement, arrest development and eventually population extinction (Fields, 1992, Wellheiser, 1992, Bhargava *et al.*, 2007). It has been estimated that in stored-product insects temperatures between 40 - 50^{o}C cause death in days, between 50-60^{o}C within hours and above 60^{o}C within minutes (Fields, 2006, Hammond, 2015).

The mortality of insects at extreme temperatures is given in terms of temperature and duration of exposure (Fields, 1992, Denlinger & Yocum, 1998, Beckett *et al.*, 2007,

Bowler & Terblanche, 2008). Insect performance decreases more rapidly above than below the optimum temperature, meaning that a shorter time is needed to reach mortality at high than at low temperatures (Denlinger & Yocum, 1998). The lethal times for 50 and 95% (LT_{50}, LT_{95}) of the most heat-tolerant life stage are the most commonly estimated to provide recommendations for pest control (Fields, 1992, Robertson *et al.*, 2007). For application in quarantine security (i.e., to avoid the introduction of exotic pests into a country), the United States and other countries require the estimation of the times necessary to kill 99.9968% (Probit 9) of the population, i.e., survival of 32/1,000,000 (Robertson *et al.*, 2007). To create effective protocols and decrease the cost of heat treatment, studies to control *T. granarium* at high temperatures must examine different life stages of the insect, a wide range of temperatures and exposure times, and standardization of the analytical methods to determine the lethal time for 50 and 95% of the population (LT_{50} and LT_{95}) (Wilches *et al.*, 2016).

A key feature of *T. granarium* that has contributed to its status as a pest and quarantine insect is the ability of its larvae to undergo facultative diapause (Burges, 1962a, Nair & Desai, 1972). In facultative diapause, *T. granarium* mature larvae feed and occasionally molt (Denlinger, 1991, Bell, 1994). Diapause in *T. granarium* is initiated and maintained by external cues, which include exposure to low temperatures, inadequate food, and rearing in isolation or crowding (Burges, 1962a, Nair & Desai, 1972). The termination of diapause in this insect can be spontaneous or follow changes in density of larvae, a rapid increase in temperature or photoperiod (Loschiavo, 1960, Burges, 1961, Beck, 1971b, Beck, 1971a, Wright & Cartledge, 1994). Diapause increases the tolerance of *T.*

granarium to insecticides (e.g., phosphine), starvation, extreme temperatures and dryness (Lindgren & Vincent, 1959, Bell *et al.*, 1984).

In this chapter, I report results of two experiments that together identify the combination of time and high temperature necessary to control *T. granarium*. The first experiment directly determined the most heat-tolerant life stage by exposing all life stages to 45^{o}C for different durations. The second experiment determined the LT_{50}, LT_{95}, LT_{99} and Probit 9 values for the most heat-tolerant life stage (diapausing larvae) when exposed to 45, 50, 55 and 60^{o}C. It also assessed the percentage of individuals terminating diapause after removal from the heat treatments.

Methods

Study species and life stages tested

Individuals collected from stored wheat in a Pakistani grain market were used to first establish a colony of *T. granarium* in 2011 at the Grain Research Laboratory, Training and Storage Management Cell (Department of Agricultural Entomology, University of Agriculture, Faisalabad, Pakistan). Larvae from this colony were used to start a second colony in 2013 at the Insect-Microbial Containment Facility (Lethbridge Research and Development Center, Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada). The larvae were imported into Canada with permit number P-2013-01610 issued by the Canadian Food Inspection Agency. The culture was reared in 4-liter jars, each containing 200 g of a mixture of ground dog food (Purina® Dog Chow®, Mississauga, Ontario, Canada), instant skim milk powder (Carnation® Milk, Markham, Ontario, Canada), wheat germ and Brewer's yeast (MP Biomedicals, Santa Ana, California, United States)

(ratio by weight of diet was: 47.5, 17.5, 17.5 and 17.5%, respectively) (Abdelghany *et al.*, 2015). Jars were kept at 30 ± 2°C, 10-30% RH, in 14 h light and 10 h dark conditions. All the experiments were done using the same diet that was used for rearing.

Life stages used in experiments included eggs, pupae, unsexed adults, non-diapausing larvae, and diapausing larvae. Eggs were removed with a fine-haired paint brush from Petri-dishes (100 mm diameter, 15 mm height) in which 20 adults had been placed five days previously. Pupae and adults were obtained directly from the colony. Non-diapausing larvae were obtained by rearing individuals at low densities (25 larvae/6 g of diet) for 23 ± 2 d after hatching. Diapausing larvae were obtained by rearing individuals at high densities (38, 55 or 73 larvae/g of diet as for Chapter 1-Experiment 1 results) for 60 ± 2 d after hatching (Nair & Desai, 1972).

Experiment 1: Determination of the most heat-tolerant life stage

Individuals (n = 20) from each life stage were placed in glass vials (2.8 cm diameter, 8 cm in height) with 3 g of diet, and held at 45°C in an oven (Fisher Scientific™, Isotemp™, Toronto, Ontario, Canada). Durations of exposure were based on results from previous studies (Table 1.3); i.e., eggs (0, 2, 6, 12, 18, 24 h of exposure), diapausing and non-diapausing larvae (0, 1, 2, 3, 4, 7 d), pupae and adults (0, 12, 18, 24, 48, 96 h). These periods of time were selected according to the results of previous studies that examined the time required to achieve 0, 50, and 100% of mortality at 45°C (Table 1.2). After removal from 45°C, individuals were held at 30°C for five days. Mortality was subsequently assessed by observing eggs and pupae for eclosion, and larvae and adults for movement.

The tests with diapausing larvae and other life stages at 45°C were done in August 2015. The diapausing larvae were chosen as the most tolerant life stage and used in Experiment 2 on February 2016. The results of mortality for diapausing larvae in Experiment 2 (February 2016) differed from the results of Experiment 1 (August 2015). To confirm the results, I repeated the test with diapausing larvae in May 2016.

Experiment 2: Heat tolerance of diapausing larvae

The goal of this experiment was to assess the time necessary to kill 50 (LT_{50}), 95 (LT_{95}), 99 (LT_{99}), and 99.9968% (Probit 9) of the population of the most heat-tolerant life stage (diapausing larvae) at 45, 50, 55, and 60°C (temperatures commonly used for control of insects in grain). Diapausing larvae were placed in glass vials (2.8 cm diameter, 8 cm height, n = 20 larvae/vial) with 3 g of diet. The vials were then exposed in an convection oven (Fisher Scientific™, Isotemp™, Toronto, Ontario, Canada) to different time-temperature combinations (n = 3 per each time-temperature combination): 45°C (1, 2, 3, 4, 6, and 6.5 d); 50°C (1, 2, 2.5, 3, 3.5, and 4 h); 55°C (15, 30, 45, 50, 55, and 60 min); and 60°C (10, 20, 30, 35, 40, and 50 min). After each time, mortality was assessed as described for larvae in Experiment 1. After 15 days, the termination of diapause was assessed as the percentage of larvae that survived the exposure and pupated (Burges, 1962b, Nair & Desai, 1973b).

Data analyses

The time-percent-of-mortality relationship at a given temperature has a sigmoidal shape; to estimate the lethal times it is transformed into a linear relationship with probit (normal distribution) or logit analysis (Fields, 1992, Robertson *et al.*, 2007). In the current study,

the probit analysis without logarithmic transformation of the time of exposure was used to calculate the LT_{50} and LT_{95} for each life stage or temperature. In Experiment 2, Probit analysis without logarithmic transformation was used to calculate the time required to kill 99% (LT_{99}) and 99.9968% (Probit 9) (Robertson *et al.*, 2007). Statistical analyses were performed in PoloPlus 2.0, LeOra Software (Finney, 1971).

Results

Experiment 1: Determination of the most heat-tolerant life stage

Diapausing larvae (May 2016) were the most heat-tolerant when compared to all life stages, ranging from 1.5 times more heat-tolerant than non-diapausing larvae to 11.5 times more heat-tolerant than eggs (Table 3.1). Based on LT_{50} values at 45^{o}C, the heat tolerance of different life stages in descending order was: diapausing larvae > non-diapausing larvae > adults > pupae > eggs. The larvae of *T. granarium* are the most heat-tolerant life stage of *T. granarium* and diapause seem to increase heat tolerance even further. Diapause caused a 1.5-fold increase in the LT_{50} of non-diapausing larvae when compared to the results of May 2016 (Table 3.1). The results for diapausing larvae performed in August 2015, do not show an increase in the mean LT_{50} when compared to non-diapausing larvae. The non-diapausing larvae can be controlled at 45^{o}C by an exposure of 4.4 d compared to 8 d for diapausing larvae (Table 3.1- LT_{95}). The X^2 goodness-of-fit statistic versus degrees of freedom for each life stage indicates that the probit model adequately fitted the data (Table 3.1, $P > 0.05$).

The LT_{50} and LT_{95} for diapausing larvae on May 2016 were different than experiments performed in August 2015 (Table 3.1), but not different from results of Experiment 2

(February 2016) (Table 3.2). For this reason, the results of August 2015 are shown in Table 3.1, but comparisons among tolerance of life stages were done based on the results of May 2016. Further tests are needed to confirm that diapausing larvae are actually more heat-tolerant than non-diapausing larvae.

Experiment 2: Heat tolerance of diapausing larvae

The most heat-tolerant life stage, diapausing larvae, can be controlled with exposure at temperatures higher than 45^{o}C. Exposures between 1.5 - 8 h suffice for the control (LT_{99} upper confidence interval limit) of diapausing larvae at temperatures between 50 - 60^{o}C (Table 3.2). The value of probit 9 was calculated to provide recommendations for quarantine uses. In quarantine uses, exposures between 2 - 12 h are necessary to cause 99.9968% (Probit 9) mortality at temperatures of 50 - 60^{o}C (Table 3.2), but more replication is needed to obtain more precise estimates.

The percent of larvae that terminated diapause was lower at longer exposure times (Fig. 3.1). The induction of diapause in larvae was obtained by rearing at high densities (38, 55 or 73 larvae/g of diet), and held at 30^{o}C for 60 d. Approximately 50% (SEM = 3.07) of the resulting diapausing larvae, that were not exposed to high temperatures, broke diapause (pupated within 15 d) when placed on fresh diet and lower densities (7 larvae/g). When diapausing larvae were held at high temperatures, the percentage of larvae that broke diapause (pupated) decreased with time held at high temperatures. This result is most evident at 50^{o}C (Fig. 3.1).

Discussion

The results of the current study validate the need to test all life stages of an insect being targeted for control using high temperatures. The most heat-susceptible life stages were eggs and pupae, which agrees with results for the congeneric *T. variabile* (Wright, 2002). Some authors suggest eggs are generally the most heat-susceptible life stage in stored-product insects (Hammond, 2015). However, there are some examples where the eggs are the most heat tolerant life stage: *Lasioderma serricorne* (Coleoptera: Anobiidae) (Yu, 2008), *Liposcelis bostrychophila*, *Liposcelis decolor*, and *Liposcelis paeta* (Psocoptera: Liposcelididae) (Beckett & Morton, 2003a). Therefore, heat tolerance of different life stages varies among species and must be assessed for each species independently. Differences in the heat tolerance among life stages are due to ontogenetic, physiological, and ecological differences among them (Baust & Rojas, 1985, Denlinger & Lee 1998). Eggs and pupae of *T. granarium* might be more sensitive to heat because they have a higher occurrence of cell growth than other life stages (Knight *et al.*, 1986, Lee, 1991, Mason & Strait, 1998).

Physiological changes during diapause can increase heat tolerance in insects (Denlinger & Yocum, 1998). Reduced metabolism, arrested cellular activities, and the production of stress proteins during diapause allow insects to better withstand thermal and other ecological stresses. According to the results from tests performed on August 2015, there is no difference in the heat tolerance between diapausing and non-diapausing larvae (Table 3.1). However, on subsequent tests performed on February 2016 and May 2016, larvae in diapause survived 2.5 times longer than non-diapausing larvae (Table 3.1). These latter results agree with previous studies that suggest diapause is a factor increasing

heat tolerance in *T. granarium.* Battu *et al.* (1975) concluded that at 45^{o}C, an exposure of 4 d and 6 d is necessary to control non-diapausing larvae and diapausing larvae, respectively. Diapause also increased the LT_{50} of *T. granarium* by 40%. Further studies are necessary to clarify the effect of diapause on heat tolerance in *T. granarium.*

The development of methods of control using high temperatures should target the most tolerant life stage of the insect, which generally is the stage in diapause (Howe, 1962, Fields, 1992). In *T. granarium*, I have shown that diapausing larvae are the most heat-tolerant life stage at 45^{o}C. The relation between diapause and heat tolerance seems to be coincidental, and a consequence of physiological changes occurring in diapause that might also increase heat tolerance (Masaki, 1980). For example, pupae in diapause of the fly *Sarcophaga crassipalpis* have a puparium coated with twice the amount of hydrocarbons than the non-diapausing pupae. The higher quantity of hydrocarbons prevents water loss in the pupae in diapause and consequently increases its heat tolerance (Yoder *et al.*, 1995).

Differences on the effect of diapause among species and generations of the same species might be due to the conditions used to induce diapause. Similar to *T. granarium*, diapause increases heat tolerance in other stored product insects; e.g., in *Ephestia elutella* (Lepidoptera: Pyralidae) and *Plodia interpunctella*, larvae in diapause have a higher tolerance at 46^{o}C than non-diapausing larvae (Johnson *et al.*, 2003). However, in the congeneric *T. variabile*, non-diapausing larvae were found to be the most heat-tolerant life stage at 56^{o}C and more tolerant than diapausing larvae (Wright, 2002). Diapause of *T. variabile* is induced by rearing in isolation (Hagstrum & Silhacek, 1980, Wright, 2002, Abdelghany *et al.*, 2015). Conversely, the crowded conditions used to induce diapause in

T. granarium and *P. interpuctella* might trigger the production of proteins involved in thermal tolerance (Sørensen *et al.*, 2003). For example, larval crowding in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) induces the expression of heat-shock proteins, and increases adult longevity and resistance to thermal stress (Sørensen & Loeschcke, 2001).

The differences in the heat tolerance of diapausing larvae on different dates might be due to high intra-population variability in the diapause intensity. The differences in the lethal times calculated for diapausing larvae tested at 45^{o}C in August 2015 and May 2016 (Table 3.1) might be due to a deeper state of diapause in the latter group (Burges, 1962b, Tauber & Tauber, 1976, Masaki, 2002). Larvae deeper in diapause have a lower metabolism, higher production of stress proteins and consequently an increased tolerance to extreme conditions (Sinclair *et al.*, 2013). This deeper state of diapause might have been caused by differences in relative humidity at rearing (Denlinger & Lee 1998, Subramanyam *et al.*, 2011). The heat tolerance in insects can also be acquired through genetic adaptation, long exposure to high temperature and rapid heat hardening (Denlinger & Yocum, 1998). In this case, heat adaptation is less likely because of the low genetic variability of the laboratory colony (Nair & Desai, 1973a). However, heat-adaptation could happen in field conditions, and future research should consider genetic adaptation and development of heat tolerance, as well as heat tolerance related to exposure to other stresses including crowding (Denlinger & Yocum, 1998) (Subramanyam *et al.*, 2011).

Trogoderma granarium is considered one of the most heat-tolerant stored-product insect pests (Fields, 1992). Previous studies indicated that an exposure of 2 h at 50^{o}C is fatal for

all life stages of stored-product insects (Michelbacher, 1953, Evans, 1986, Fields, 1992, Phillips & Throne, 2010). However, many diapausing larvae of *T. granarium* would survive a 2 h exposure at 50°C. The control of all life stages (upper limit of LT_{99}) would require an exposure of 8 h (Table 3.2). *Rhyzoperta dominica* (Coleoptera: Bostrychidae) is the only other stored-product insect known to have a high heat tolerance (Mookherjee *et al.*, 1968). The upper limit of LT_{50} for *R. dominica* is 3 h at 50°C or 31 h at 45°C (Beckett *et al.*, 1998, Beckett & Morton, 2003b), whereas for *T. granarium* these values correspond to 4 and 161 h respectively (Table 3.2). The high heat tolerance of *T. granarium* might reflect its adaptations to avoid desiccation, which is the mechanism of heat-related damage at temperatures below 50°C (Fields, 1992, Denlinger & Yocum, 1998). The congeneric *T. variabile* is more heat-susceptible and can be controlled by an exposure of 5 min at 56°C (Wright, 2002). This result is not unexpected, given that *T. granarium* is endemic to hot and dry environments, whereas *T. variabile* has a Nearctic origin (Hinton, 1945, Banks, 1977, Eliopoulos, 2013).

The Probit 9 values estimated in this study should be considered approximations, but can be used in confirmatory tests for design quarantine treatments. The Probit 9 mean values calculated for *T. granarium* were 2 h for 60°C (Table 3.2). These exposure times are longer than the recommendations for the post-entry quarantine treatment of commodities and transport vehicles (MBTOC, 2014). However, the recommendations of the USDA were done based on information that is more than 20 years old, variable among studies, and were not calculated using the minimum of 93,613 insects with no survivors needed to calculate Probit 9 mortality at the 95% confidence level (Follett & Neven, 2006). Although we did not test the required quantity of insects, the Probit 9 values estimated in

this study can be used in confirmatory tests by a specialized quarantine laboratory with the capacity to produce large numbers of individuals in order review the current recommendations (Robertson *et al.*, 2007). The validity of the Probit 9 measure has been criticized, but is still an obligatory requirement to provide recommendations for the control of quarantine pests (Robertson *et al.*, 2007). Additional tests with *T. granarium* at 65^{o}C would be needed to determine if these recommendations are enough for the control of this pest. For the design of recommendations for quarantine treatments, current data should be used in the development of mathematical models that consider the commodity or structure to be treated, the infestation rate of the commodity, and the probability of establishment of the pest (Jian *et al.*, 2013, Follett & McQuate, 2001, Landolt *et al.*, 1984).

The percentage of *T. granarium* diapausing larvae that broke diapause after heat treatment diminished with longer durations of exposure. Diapausing individuals were defined as live larvae that did not pupate. About 50% of diapausing larvae that were not exposed to high temperatures broke diapause after being placed on new food and lower larval densities. Diapausing larvae that were exposed to high temperature were less likely to break diapause (pupate after 15 d) with increasing durations at high temperature (Fig. 3.1). This trend could be interpreted as high temperature causing insects to go deeper into diapause. Individuals deeper in diapause need a higher intensity of stimuli to break diapause (pupation) and have higher tolerance to extreme conditions (Burges, 1962b, Tauber & Tauber, 1976, Masaki, 2002).This diapause intensity is represented by variable durations of diapause in individuals of the same population under the same rearing conditions (Tauber & Tauber, 1976, Masaki, 2002). These results agree previous studies

that state that diapause intensity has high intra-population variability, and can be affected by various factors; e.g., sex, geographic strain, and the influence of pre-diapause environmental conditions (Tauber & Tauber, 1976, Masaki, 2002). An alternative hypothesis is that higher temperatures and longer exposures cause more damage to the larvae that prevents them from pupating, yet does not kill them; e.g. altering the structure of macromolecules and the rate of metabolic reactions (Nedved, 1999, Hansen *et al.*, 2011). Further experiments are needed to distinguish these alternative hypotheses.

The ability to undergo diapause is beneficial in the stored-product environment, because it allows *T. granarium* to tolerate suboptimal conditions where the food supply and available mates are sporadic and unpredictable (Beck, 1973, Armes, 1988, Armes, 1990). This insect does not fly and hence has low dispersal ability (Burges, 1962a). The diapause state allows individuals to synchronize development, to withstand starvation and to survive adverse conditions including extreme temperatures and desiccation (Burges, 1962a, Tauber *et al.*, 1986, Pullin, 1996). The diapause stage terminates with optimal conditions for growth and reproduction. Adults of *T. granarium* that have completed diapause have been reported to be larger and lay more eggs than their non-diapausing counterparts, which promotes a rapid increase in the post-diapause population (Karnavar, 1972). Further research needs to be conducted to identify triggers for induction and termination of diapause and their effects on diapause intensity and heat tolerance. It should also assess the effect of diapause and heat treatment on fecundity, and focus on developing methods to better distinguish between diapausing and non-diapausing larvae.

In addition to mortality, exposure to high temperatures can cause developmental abnormalities (phenocopies) and loss of fecundity (Denlinger & Yocum, 1998). For

example, exposure to moderately high temperature (35 - 45^{o}C) of *Drosophila* individuals during embryogenesis or pupal metamorphosis caused anomalies of development (Lindquist, 1986, Denlinger & Yocum, 1998). This phenomenon is linked to the disruption of enzymatic reactions or hormone production necessary for development, and causes a reduction of fitness in the adult fly (Lindquist, 1986, Denlinger & Yocum, 1998). Future research should consider non-lethal consequences of high temperatures and the importance of diapause on heat tolerance to avoid mortality and developmental abnormalities.

Conclusions

1. Diapause may increase heat tolerance in *T. granarium,* but this needs to be confirmed with more experiments. Further research needs to be conducted to identify triggers for induction and termination of diapause, to clarify the effect of diapause and heat treatment on fecundity, and to develop methods to better distinguish between diapausing and non-diapausing larvae.

2. *Trogoderma granarium* has a high heat tolerance, and an exposure of 1.2 h at 60^{o}C is recommended to control (Probit 9) the most heat-tolerant life stage. The termination of diapause in diapausing larvae after heat treatment depends on the temperature-time combination at which the larvae were exposed. Future studies should be done to develop temperature-time- mortality models to control *T. granarium* and to assess the effect of heat treatment on the quality of the treated product.

3. *Trogoderma granarium* is able to terminate diapause and continue its development after exposure to elevated temperatures. However, it is important to study the effects of

heat-treatment and diapause on the fitness of the adults to determine their ability to increase the population.

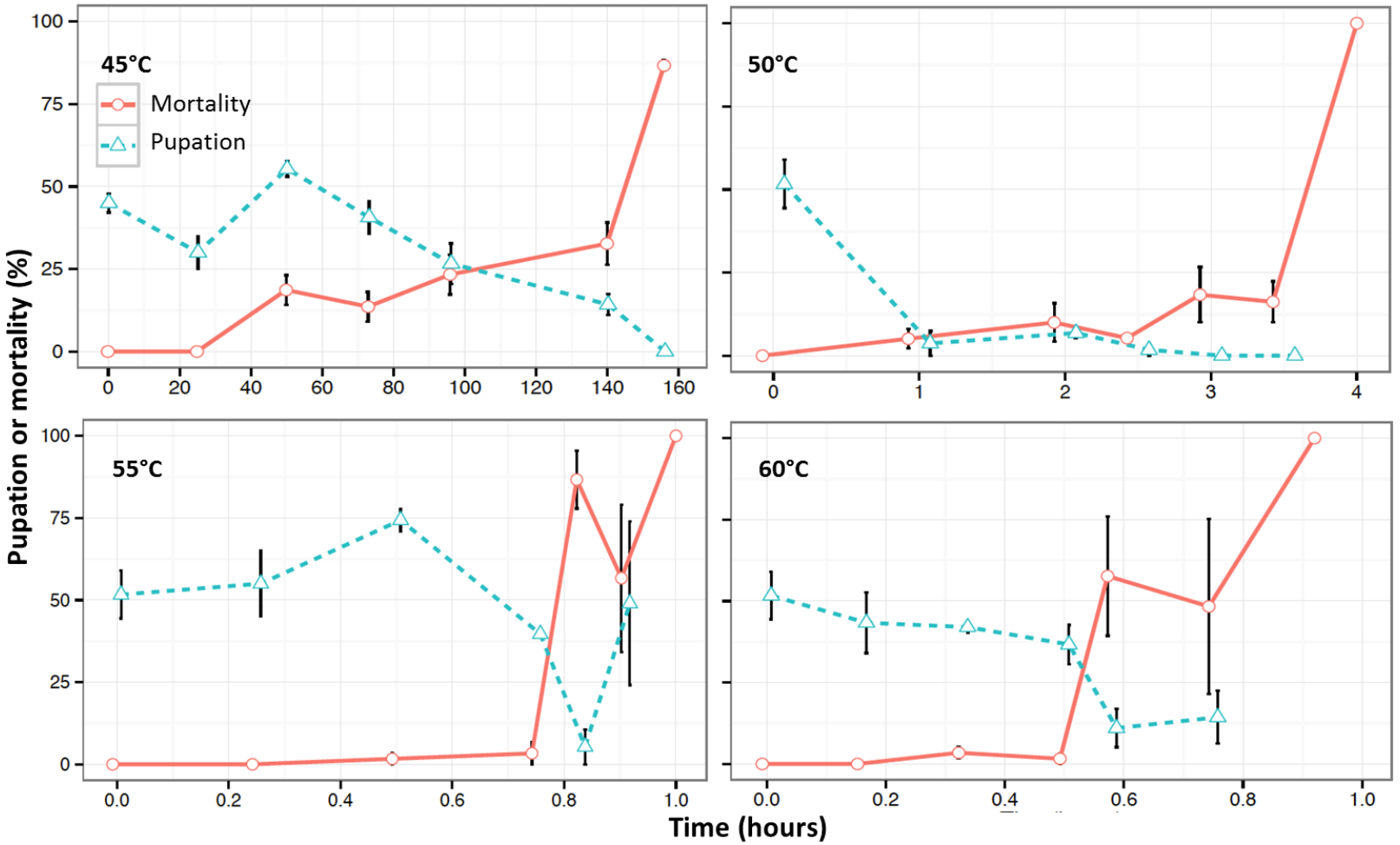


Figure 3.1 Effect of temperature and exposure time on mortality of diapausing larvae and pupation of survivors 15 days after heat treatment (break of diapause). Each value is the mean (± SE) of three samples each containing 20 diapausing larvae at the onset of exposure.

Table 3.1 Experiment 1 - Lethal time (LT) in days to kill 50 or 95% of the population of each life stage at 45°C (*n* = 3 vials with 20 individuals per duration). Differences between the data of Experiment 1 (August 2015) and Experiment 2 required repeating the test on May 2016 for confirmation of the data.

Life stage	Diapause	LT_{50} (days) (95% CI)	LT_{95} (days) (95% CI)	Slope ± SE	Intercept ± SE	X^2 (df)
Eggs[1]	No	0.40 (0.18 - 0.52)	0.71 (0.59 – 0.94)	5.23±0.91	-2.08±0.55	16.19 (10)
Larvae[1]	No	2 (1– 3)	4.4 (3 – 10)	0.67±0.08	-1.33±0.18	98.7 (14)
Larvae[1]	Yes	1.7 (1.6 – 1.8)	2.8 (2.7 – 3.1)	1.40±0.12	-2.38±0.25	17.96 (23)
Larvae [2]	Yes	5 (4.8 – 5.5)	8 (7.4 – 9.2)	0.55±0.06	-2.77±0.26	14.98 (16)
Larvae[3]	Yes	5.7 (5 – 6.7)	10 (8.6 – 13.3)	0.37±0.04	-2.13±0.20	57.18 (19)
Pupae[1]	No	1 (0.95 – 1.1)	1.8 (1.6 – 2.0)	2.28±0.18	-2.38±0.18	49.30 (29)
Adults[1]	No	1.4 (1.2 – 1.5)	1.9 (1.7 – 2.4)	2.90±0.26	-3.94±0.36	80.49 (18)

[1] Test of August 2015.

[2] Test of May 2016.

[3] Test of February 2016

Table 3.2 Experiment 2 - LT_{50}, LT_{95}, LT_{99} and Probit 9 values for diapausing larvae of *T. granarium* at different temperatures (Feb 2016).

Temperature (± 1°C)	**LT_{50} (h) (95% CI)**	**LT_{95} (h) (95% CI)**	**LT_{99} (h) (95% CI)**	**Probit 9 (h) (95% CI)**	**Slope ± SE**	**Intercept ± SE**	**X^2 (df)**
45	137 (121 – 161)	244 (206.9 – 322)	288 (240– 391)	397 (320- 563)	0.015±0.002	-2.12±0.19	57.34 (19)
50	3.5 (3 – 4)	5 (4 – 7)	6 (5 – 8)	7 (6 – 12)	1.034±0.1	-3.67±0.332	127.51 (22)
55	0.8 (0.7 – 0.9)	1 (0.9 – 1.3)	1.1 (1 – 1.5)	1.2 (1 – 2)	9.39±1.07	-7.76±0.91	147.14 (19)
60	0.6 (0.5 – 0.7)	0.9 (0.8 – 1.2)	1 (0.8 – 1.4)	1.2 (1 – 2)	6.56±0.60	-4.02±0.38	180.93 (22)

CHAPTER 4: THE MICROBIOME OF *Trogoderma granarium*

Abstract

Trogoderma granarium (Coleoptera: Dermestidae) is a pest of stored grain present in North Africa, Europe and Asia, but for much of the rest of the world it has quarantine status. The pest status of *T. granarium* is enhanced by the ability of its larvae to undergo diapause, which increases its tolerance to adverse conditions including insecticides and extreme temperatures. The ability of insects to tolerate extreme conditions can be influenced by their associated bacterial community (the microbiome), and understanding this relationship may lead to improved methods of pest control. However, the microbiome of *T. granarium* is still not known. In this chapter, I used next-generation sequencing methods to: 1) compare the microbiomes of *T. granarium* and its congener *T. variable*, 2) assess the effect of life stage on the microbiome of *T. granarium*, and 3) assess the effect of extreme temperatures on the microbiome of *T. granarium*. In these studies, I identified that the core microbiomes of *T. granarium* and *T. variable* are similar in composition, but the relative abundance of *Spiroplasma* is significantly higher in *T. granarium* (higher than 90%) than in *T. variable* (lower than 3%). This also suggests a possible key association between *T. granarium* and *Spiroplasma* bacteria. The microbiome of *T. granarium* was affected by life stage; evidence for the vertical transmission of *Spiroplasma* in *T. granarium* was supported by its presence in the eggs. High temperatures significantly reduced the relative abundance of *Spiroplasma* in *T. granarium*, but an effect of low temperatures on its microbiome was not detected. Further studies are needed to understand the association of *Spiroplasma* and *T. granarium*, and to improve or develop new methods of control against this insect pest.

Introduction

Trogoderma granarium is among the most important invasive and destructive pests of stored agricultural products (Eliopoulos, 2013). This pest is native to India, but it has been introduced in Africa, Asia, and Europe (OEPP/EPPO, 2013, OEPP/EPPO, 2015). It is considered a quarantine insect for Canada, United States, Australia, New Zealand, and South America. If introduced into these areas, it would cause a high economic impact (OEPP/EPPO, 2013, OEPP/EPPO, 2015). *Trogoderma granarium* is a xerophilous insect (able to thrive in dry environments) that is able to survive feeding on food with water content below 10% (Zhantiev, 2009). For this reason, this insect can survive feeding on more than 96 commodities of animal or vegetal origin including textiles and dead animals (Eliopoulos, 2013). The invasive pest status of *T. granarium* lies in the damage it can cause to the commodities, and in the resilience of its diapausing larvae (Lindgren & Vincent, 1959). The larvae of *T. granarium* can enter a state of developmental arrest termed facultative diapause following detrimental environmental cues (low temperature, lack of food or high densities) (Tauber *et al.*, 1986, Koštál, 2006). Larvae of *T. granarium* in diapause have a high tolerance to starvation, insecticides, extreme conditions and temperatures making it difficult to eradicate (Lindgren & Vincent, 1959, Bell *et al.*, 1984, Wilches *et al.*, 2014). Although it has not been assessed, the resilience and high tolerance of *T. granarium* to extreme conditions (Wilches *et al.*, 2016) might be due to its associated microbial community.

The microbial community and microbial genomes (microbiome) associated with insects can have harmful, neutral or beneficial effects on the physiological and ecological traits of their hosts (Sachs *et al.*, 2011). The microbiome can improve the host's ability to

explore new habitats, provide resistance against natural enemies, induce resistance to pesticides, produce nutrients for the host, influence host reproduction, suppress the actions of deleterious genes in the host genome, and increase tolerance to extreme conditions including extreme temperatures (Zindel *et al.*, 2011, Wernegreen, 2012). The minimum community of microbes that is essential for the good functioning of the host is termed the core microbiome (Shade & Handelsman, 2012). The taxa that are part of the core microbiome are common to most of the hosts in a particular habitat, and are thought to be the key host/microbe associations because they fulfill a functional niche within the community (Meriweather *et al.*, 2013, Shade & Handelsman, 2012). Beneficial microorganisms in the microbiome can help insects to adapt to changing environments and increase their fitness (McFall-Ngai *et al.*, 2013, Moran & Sloan, 2015). For example, the presence of *Rickettsia* in whiteflies (*Bemisia tabaci*) at normal rearing temperatures induces the expression of host genes required for thermotolerance, which increases host tolerance to heat shock (Brumin *et al.*, 2011). The tick, *Ixodes scapularis*, survives temperatures of -20°C due to the production of an antifreeze glycoprotein induced by its bacterial endosymbiont, *Anaplasma phagocytophillum* (Neelakanta, 2010).

The composition of the host's microbiome can be shaped by environmental temperature, interactions with other members of the microbiome, host genotype, diet, and physiological condition (Douglas, 2007, Zilber-Rosenberg & Rosenberg, 2008, Moran & Sloan, 2015). Environmental temperature is important for infection success, density, transmission, and activity of endosymbionts (Thomas & Blanford, 2003). In highly dependent insect-bacteria relationships, the bacteria often evolve to have reduced genomes, losing their DNA repair systems, and the ability to repair heat-related damage

(Wernegreen, 2012). For this reason, extreme heat can eliminate the bacterial symbiont and its effects on the host (Wernegreen, 2012). For example, infections of *Wolbachia* and *Buchnera* can be removed using high temperatures (Russell & Moran, 2006, Li *et al.*, 2014). Microbiome composition is also affected by the host's genotype and has high intra- and interspecific variability due to ecological and physiological differences (Chandler *et al.*, 2011). Insects genetically control their microbiome by regulating the expression of genes related to their immune response; mutations in a single host gene are sufficient to alter the microbiome composition (Ryu *et al.*, 2008, Lhocine *et al.*, 2008, Lee, 2008). In holometabolous insects, due to physiological and dietary differences between life stages, the microbiome changes during metamorphosis (Hammer *et al.*, 2014).

Diet can shape the microbiome, in some cases the gut microbiome has evolved as a metabolic resource for insects feeding on suboptimal diets (Dillon & Dillon, 2004, Hosokawa *et al.*, 2007, Russell *et al.*, 2009). For example, microbial taxa present in each life stage may be involved in utilizing life-stage-specific resources by providing functions related to digestion, detoxification and/or nutrient supplementation (Hosokawa *et al.*, 2006, Gosalbes *et al.*, 2010, Chandler *et al.*, 2011, Hammer *et al.*, 2014). These associations are common in insects feeding on diets that are unbalanced or difficult to digest, such as phloem or xylem sap (low in proteins and lipids), woody or keratinous materials (hard to digest, low nitrogen), blood, and grains (deficient in B vitamins) (Douglas, 2007, Fukatsu *et al.*, 2007, Gosalbes *et al.*, 2010, Russell *et al.*, 2009, Zindel *et al.*, 2011). Keratinous materials (i.e., feathers and hairs) composed mainly of cysteine, are difficult to digest and poor in amino acids and vitamins. Similarly, grain-based diets

provide insects with starch and carbohydrates, but are deficient in other nutrients and essential vitamins (e.g., vitamin B complex) (Gosalbes *et al.*, 2010). Insects feeding on grain usually harbour symbiotic bacteria with supplementation or detoxification functions (Chapter 1, Table 1.4). For example, the stored-grain pest *Stegobium paniceum* has an association with *Symbiotaphrina buchneri,* a yeast-like symbiont that produces vitamin B, sterol, and essential amino acids (Buchner, 1965, Dowd, 1989, Ashworth, 1993).

The high tolerance of *T. granarium* might be due to associations with microorganisms that could facilitate keratin digestion, supplement essential nutrients, or overcome extreme conditions, but these has not been previously assessed (Gosalbes *et al.*, 2010, Rajendran & Hajira Parveen, 2005, Fukatsu *et al.*, 2007, Eliopoulos, 2013, Wilches *et al.*, 2016). The available studies on the gut microbiota of dermestids are more than 25 years old and use techniques that do not detect uncultivable bacteria (believed to be > 90% of the microbiome) (Feldhaar & Gross, 2009). The most recent study on dermestids detected, through microscopy, a large quantity of bacterial cells and amoeba-like protozoans colonizing wool-fibre fragments' in the intestine of *Anthrenus flavipes,* a pest of textiles (Trivedi *et al.*, 1991). Other dermestids might also have microorganisms that assist in keratin or grain digestion or provide nutrients supplementation (Trivedi *et al.*, 1991). More recent studies surveyed bacteria that affect reproduction (*Wolbachia, Cardinium, Rickettsia,* and *Spiroplasma)* in dermestids. *Wolbachia* was detected in *Attagenus unicolor*, *Anthrenus verbasci* and *Dermestes lardarius* (Li *et al.*, 2015, Zchori-Fein & Perlman, 2004); however, *Trogoderma variabile* and *T. granarium* are negative for the presence of *Wolbachia* and *Cardinium* (Chapter 1, Table 1.4). Detection of other

reproductive bacteria and their effects on dermestids has not been assessed (Zchori-Fein & Perlman, 2004, Li *et al.*, 2015).

The parameters shaping the microbiome of *T. granarium* and its interactions could provide new insights into the development or improvement of methods of control against this pest (Sinkins *et al.*, 1997, Zindel *et al.*, 2011). Microbiome-related methods of control include the use of cytoplasmic incompatibility to reduce the population of the insect, the expression of factors in the symbiont that have detrimental effects on the insect, and the removal of beneficial bacteria affecting insect survival (Zindel *et al.*, 2011, Knight & Witzgall, 2013, Minard *et al.*, 2014). In the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), the discovery of a relationship with yeasts allowed for the development of attract-and-kill techniques combining a pathogenic virus and symbionts into sprays (Witzgall *et al.*, 2012, Knight & Witzgall, 2013). Before this, the use of pathogenic viruses had been hampered by the low intake and short life of the virus (Knight & Witzgall, 2013).

The objective of this chapter is to investigate the composition and factors that can affect the microbiome of *T. granarium*. This is assessed in three studies using next-generation Illumina sequencing of the 16S rRNA gene. Study 1 assessed interspecific differences in the bacterial community associated with adults of *T. granarium* and its congeneric, *T. variabile*. Study 2 evaluated the differences in the microbiome composition of *T. granarium* associated with different life stages (eggs, larvae, diapausing larvae, acclimated-diapausing larvae, pupae, and adults). Study 3 evaluated the effect of extreme temperatures on the microbiome composition of *T. granarium* adults.

Methods

Study species and life stages tested

Individuals collected from stored wheat in a Pakistani grain market were used to first establish a colony of *T. granarium* in 2011 at the Grain Research Laboratory, Training and Storage Management Cell, Department of Agricultural Entomology, University of Agriculture, Faisalabad, Pakistan. Larvae from this colony were used to start a second colony in 2013 at the Insect-Microbial Containment Facility (Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada). The larvae were imported into Canada with permit number P-2013-01610 issued by the Canadian Food Inspection Agency. *Trogoderma variable* were collected in 2008 from a mill at Yorkton, Saskatchewan, Canada and maintained at 25 to 28^{o}C. The insect cultures were reared in 4-litre jars on 200 g of a mixture of ground dog food (Purina® Dog Chow®, Mississauga, Ontario, Canada), instant skim milk powder (Carnation®Milk, Markham, Ontario, Canada), wheat germ, and Brewer's yeast (MP Biomedicals, Santa Ana, California, United States) (ratio by weight of food media was: 47.5, 17.5, 17.5 and 17.5%, respectively) (Abdelghany *et al.*, 2015). The rearing conditions were 30 ± 2^{o}C for *T. granarium*, 25 ± 3^{o}C for *T. variable*, 15 ± 10% R.H., in 14 h light and 10 h dark conditions. All the studies were done using the same diet mix that was used for rearing.

The life stages tested were eggs, pupae, unsexed adults, and larvae of four different states; i.e., non-diapausing non-acclimated larvae, acclimated larvae, diapausing larvae, diapausing-acclimated larvae, where acclimation was with respect to temperature. Eggs were removed with a fine-haired paint brush from Petri dishes in which 20 adults had

been placed five days previously. Pupae and adults were obtained directly from the colony. Non-diapausing larvae were obtained by rearing individuals at low densities (4 larvae/g of diet) for 23 ± 2 d after hatching, whereas larvae in diapause were obtained by rearing individuals at high densities (38, 55 or 78 larvae/g of diet) for 60 ± 2 d after hatching (modified from Nair & Desai, 1972). Larvae were acclimated by exposure to 15^{o}C for 14 d, then 10^{o}C for 14 d, and then held at 4^{o}C for 25 d (Abdelghany *et al.*, 2015).

To assess the effect of extreme temperatures, I collected unsexed adults (n = 6) that survived an exposure of 42 hr at −15^{o}C and 4 hr at 50^{o}C. The same adult samples used in Studies 1 and 2 were used as a control in Study 3 (n = 6).

Sample processing

Individuals of each comparison group were collected live and placed in 95% alcohol solution. Larval stages, adults, and pupae were surface sterilized with two washes in 2.5% sodium hypochlorite solution followed by two washes with sterile water. Eggs were surface-sterilized by a 10 min immersion in a 0.6% sodium hypochlorite solution followed by three washes with sterile water. Template DNA was extracted with the DNeasy® Blood & Tissue Kits (Qiagen®Group, USA) by using one insect per sample (n = 6 samples per life stage and per temperature treatment).

DNA extraction and sequencing

The 16S rRNA gene universal PCR primers 27F 5ˈ-AGRGTTTGATCMTGGCTCAG- 3ˈ and 519R 5ˈ-GTNTTACNGCGGCKGCTG-3ˈwith the barcode on the forward primer were used in a PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the

following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated AMPure XP® PCR purification beads (Agencourt®, USA). These pooled and purified PCR products were used to prepare a DNA library by following Illumina® TruSeq DNA® library preparation protocol (Illumina Inc.®, USA). Amplicons of the 16S rRNA were sequenced at Mr DNA (Shallowater, TX, USA) on the Illumina MiSeq platform.

Data analyses

Filtering and denoising of the sequences was done in QIIME v. 1.8.0 (Caporaso *et al.*, 2010) by removing sequences with ambiguous base calls, sequences with homopolymer runs exceeding 6 bp, chimeras, and sequences shorter than 200 bp. The sequences were clustered into operational taxonomic units (OTUs) at the 97% similarity level (Edgar, 2010, Hammer *et al.*, 2014) using BLASTn against the February 2011 release of the Greengenes database (DeSantis *et al.*, 2006) with remaining sequences clustered *de novo.* The core microbiome for *T. granarium* and adults of *T. variabile* was calculated as the OTUs that were present in 95% of the samples (Meriweather *et al.*, 2013, Shade & Handelsman, 2012). The most abundant genera (> 3% in at least half of the samples/treatment) are summarized in Table 4.1 for Study 1, Table 4.3 for Study 2 and Table 4.5 for Study 3.

Alpha (within-sample) and beta (among-sample) diversity were calculated using the relative abundance for each OTU. The OTU table was randomly subsampled with 10 iterations at different depths (total number of sequences) and rarefied tables were generated using the maximum number of sequences as the rarefaction level. To standardize the data and make comparisons among samples, alpha and beta diversity corresponding to the minimum number of sequences found in a sample was used for each study. Four different metrics were calculated to estimate alpha diversity; i.e., the number of observed OTUs (observed richness), Shannon index (considers richness and evenness) (Shannon & Weaver, 1949), Chao1 (metric for richness) (Chao, 1984), and Faith's phylogenetic diversity (Faith, 1992).

To assess the effect of treatment on microbial communities, distance matrices were calculated using weighted and unweighted UniFrac analysis. UniFrac analysis is a phylogenetically aware measure of beta-diversity that generates a matrix of pairwise differences between the samples (Lozupone & Knight, 2005). There are two types of UniFrac analysis, weighted and unweighted. Unweighted UniFrac analysis is qualitative and compares groups based on presence/absence of OTUs. In contrast, weighted UniFrac analysis is quantitative and takes into account differences in taxon abundance among samples to calculate the distance matrix (Lozupone & Knight, 2005). The unweighted or weighted distance matrix was used in principal coordinate analysis (PCoA) to compute principal coordinates that represent the difference in the microbiomes among samples. In addition, to test for significant differences in the microbiomes among the samples, I used a permutational multivariate analysis of variance (Adonis) (Anderson, 2001). This analysis uses the calculated distance matrices (weighted or unweighted) and 1000

permutations of the observations, reorganized across the treatment groups to test for differences among the treatments and to quantify the amount of variation that can be explained by the treatments (R^2) (Anderson, 2001).

Because the distribution of the data differed among studies, different approaches were used to assess the effect of treatment on the relative abundance of the most abundant genera and on alpha diversity. In Study 1, data that matched or failed to match parametric assumptions were analysed using two-sample t-tests and Wilcoxon rank sum tests, respectively. In Studies 2 and 3, the Kruskal-Wallis test was used to test significant differences followed by pairwise multiple comparisons using Tukey-Kramer for post-hoc comparisons on ranked data (Nemenyi-test).

Statistical analysis was performed using the statistical package R (R-Development-Core-Team, 2011) and QIIME v. 1.8.0 (Caporaso *et al.*, 2010).

Results

After filtering and denoising of the raw sequences, there was an average of 17961.89 (SD = 2857.80) 200 bp sequences per sample (n = 54). These sequences represented a total of 1359 bacterial OTUs that comprised 15 phyla, 64 orders, 110 families, and 138 genera.

Preliminary examination of alpha diversity (Shannon index (H')) identified four outliers (samples from adults at 30^{o}C were used in the three studies) among samples of *T. granarium* (Fig. 4.1A-C). For these outliers, fewer than 2% of the sequences were identified as *Spiroplasma* spp., versus more than 98% for all other samples from this host. The lower quantity of *Spiroplasma* spp. in these outliers "unmasked" the presence of less abundant species leading to inflated measures of alpha diversity. Conversely, the outlier

in non-diapausing non-acclimated larvae had a very high relative abundance of sequences identified as *Spiroplasma* spp., 99.6% compared to the other samples 0.67 % and caused the opposite effect in alpha diversity. These outliers were therefore removed prior to analyses.

Study 1: The microbiome of T. granarium *and* T. variabile

Microbiome composition

The average number of sequences obtained from *T. granarium* was significantly higher than the average obtained from *T. variabile* (two-sample t-test; t (9) = 3.57; $P = 0.006$, $n = 11$). The average number of sequences for *T. granarium* ($n = 5$) was 19943 ± SEM 7.83 sequences per sample with a minimum of 19919 and a maximum of 19965 sequences. The average number of sequences for *T. variabile* ($n = 6$) was 14250 ± 1441 sequences per sample with a minimum of 9223 and a maximum of 19049 sequences.

The core microbiome, defined as the OTUs present in > 95% of the samples for the adults of *T. variabile* and for all life stages of *T. granarium*, was similar between the two host species (Table 4.1). For *T. granarium*, the core microbiome comprised *Halospirulina*, *Pseudomonas*, and *Spiroplasma*. The core microbiome of *T. variabile* adults included these same genera plus an unidentified genus from the family Solibacteraceae. *Spiroplasma* were more abundant in *T. granarium* ($n = 5$) than in *T. variabile* ($n = 6$) (Wilcoxon rank sum test; $W = 30$; $P = 0.004$). In contrast, *Halospirulina* (Wilcoxon rank sum test; $W = 0$; $P = 0.004$; $n = 11$) and *Pseudomonas* (Wilcoxon rank sum test; $W = 0$; $P = 0.004$; $n = 11$) were more abundant in *T. variabile*. Genera represented by no more than

3% of sequences, when combined (other genera in Table 4.1), were also more abundant in *T. variabile* (Wilcoxon rank sum test, $W = 0$; $P = 0.004$; $n = 11$).

Alpha diversity: Diversity within the samples

The microbiome of *T. variabile* ($n = 6$) had a higher diversity than the microbiome of *T. granarium* ($n = 5$) (Table 4.2). The Shannon index (evenness and richness) (Wilcoxon rank sum test; $W = 0$; $P < 0.001$; $n = 11$), Faith's phylogenetic diversity (two-sample t-test; $t\ (9) = -5.96$; $P < 0.001$; $n = 1$) and number of observed OTUs (two-sample t-test; $t\ (9) = -8.4$; $P < 0.001$; $n = 11$) were significantly higher in *T. variabile* than in *T. granarium.* Conversely, the Chao1 index (estimated true richness) was not significantly different between the species (Wilcoxon rank sum test; $W = 5$; $P > 0.05$; $n = 11$).

Beta diversity: Diversity among the samples

The microbial community of *T. granarium* and *T. variabile* was significantly different in composition and abundance of bacterial taxa. There were differences in the microbiome composition between *T. granarium* and *T. variabile* (Adonis; $F_{1, 9} = 3.17$; $R^2 = 0.26$; $P = 0.005$; $n = 11$), 26% of this variation could be explained by the host species. The microbiome of *T. granarium* and *T. variabile* also differed significantly when the relative abundances of their bacterial taxa are considered (Adonis; $F_{1, 9} = 24.94$; $R^2 = 0.734$; $P = 0.005$; $n = 11$), and species explained 73% of the variation. A principal coordinate analysis of the microbiome composition (Fig. 4.2A) and abundances (Fig. 4.2B) clustered the samples of *T. granarium* together and separated them from the samples of *T. variabile* by the PC1 that accounts for 73% of intersample variation (Fig. 4.2B).

Study 2: The microbiome of different life stages of T. granarium

Microbiome composition

The average number of sequences was significantly different among life stages of *T. granarium* (Kruskal-Wallis test; $X^2 = 18.58$; df = 5; $P = 0.002$).Across all life stages (n = 32), the average number of sequences obtained after filtering was 18808 (SEM = 356.9) per sample, with a minimum number of 11873 and a maximum of 19991 sequences.

The relative abundance of some of the most abundant bacterial genera (Table 4.3) also differed among life stages. There were significant differences in the relative abundances of *Spiroplasma* spp. (Kruskal-Wallis test; $X^2 = 24.9$; $df = 5$; $P < 0.001$), *Halospirulina* spp. (Kruskal-Wallis test; $X^2 = 24.02$; $df = 5$; $P < 0.001$), *Pseudomonas* spp. (Kruskal-Wallis test; $X^2 = 19.25$; $df = 5$; $P = 0.002$), *Caulobacter* spp. (Kruskal-Wallis test; $X^2 = 13.6$; $df = 5$; $P = 0.02$), *Streptococcus* spp. (Kruskal-Wallis test; $X^2 = 14.9$; $df = 5$; $P = 0.01$), and *Lactococcus spp.* (Kruskal-Wallis test; $X^2 = 11.9$; $df = 5$; $P = 0.03$) (Table 4.3). The microbiome of eggs, non-diapausing non-acclimated larvae, and diapausing-acclimated larvae also had a significantly higher relative abundance of the less-abundant genera (with a relative abundance lower than 3%) when compared to adults (Kruskal-Wallis test; $X^2 = 24.06$; $df = 5$; $P < 0.001$; post-hoc Nemenyi-test; $P \leq 0.03$) (Table 4.3).

Alpha diversity: Diversity within the samples

The diversity of the microbiome of *T. granarium* differed among life stages; i.e., eggs and non-diapausing non-acclimated larvae significantly differed from pupae and adults in their microbiome diversity (Table 4.4). Non-diapausing non-acclimated larvae had a significantly higher Shannon index (Kruskal-Wallis test; $X^2 = 24.62$; $df = 5$; $P < 0.001$;

post-hoc Nemenyi-test; $P \leq 0.02$) and a higher number of observed OTUs (Kruskal-Wallis test; $X^2 = 20.99$; $df = 5$; $P < 0.001$; post-hoc Nemenyi-test; $P \leq 0.02$) than diapausing larvae and adults. Similarly, non-diapausing non-acclimated larvae and eggs also had a significantly higher Faith's phylogenetic diversity (Kruskal-Wallis test; $X^2 = 20.6$; $df = 5$; $P < 0.001$; post-hoc Nemenyi-test; $P \leq 0.02$) and richness (Chao1) (Kruskal-Wallis test; $X^2 = 18.5$; $df = 5$; $P < 0.001$; post-hoc Nemenyi-test; $P \leq 0.03$) than adults.

Beta diversity: Diversity among the samples

The microbial community relative abundance was significantly different among life stages of *T. granarium*. There were significant differences in the composition (presence/absence of OTUs) of the microbiome among the samples (Fig. 4.3A; Adonis; $F_{5, 26} = 1.54$; $R^2 = 0.228$; P = 0.001; n= 32), and 23% of this variation can be explained by the life stage. The microbiomes of the life stages also differed significantly in the relative abundances of their bacterial taxa (Fig. 4.3B; Adonis; $F_{5, 26} = 5.82$; $R^2 = 0.528$; $P = 0.001$; $n = 32$) and life stage explained 53% of this variation. This was confirmed by plotting the first three principal coordinates of the analysis considering the relative abundances of the bacterial taxa (Fig. 4.3B), which showed that samples from larvae, eggs, and three samples of diapausing-acclimated larvae were separated from the other life stages across the PC1 axis (74.1%).

Study 3: The effect of extreme temperatures on the microbiome of T. granarium

Microbiome composition

The average number of sequences was significantly different among temperature treatments of *T. granarium* adults (Kruskal-Wallis test; $X^2 = 6.95$; df = 2; $P = 0.03$).

Across all temperatures (n = 17), the average of sequences obtained after filtering was 18062.8 ± SEM 703.23 per sample, with a minimum number of 11521 and a maximum of 19965 sequences. The highest average number of sequences for the control treatment was 19943 ± 7.83 sequences (n = 5), followed by the low (−15°C) temperature treatment (n = 6; 17947 ± 1243.96 sequences), and the high temperature treatment had the lowest average of sequences (n = 6; 16610 ± 1484.1 sequences). Significant differences were identified between the average number of sequences of the samples treated with high (50°C) temperature and the control larvae (Post-hoc Nemenyi-test; P = 0.02). The average number of sequences of the low temperature treatment was not significantly different from the other treatments (Post-hoc Nemenyi-test; $P > 0.05$).

The relative abundance of some of the most abundant bacterial genera also differed among temperature treatments (Table 4.5). The relative abundance of *Caulobacter* spp. was the only significant difference between high and low temperature treatments (Kruskal-Wallis test; X^2 = 7.5; df = 2; P = 0.03; post-hoc Nemenyi-test; P = 0.02). There were significant differences between the control treatment (30°C) (n = 5), and the high temperature treatment (50°C) (n = 6) in the relative abundances of the genera *Spiroplasma* spp. (Kruskal-Wallis test; X^2 = 6.3; df = 2; P = 0.04; post-hoc Nemenyi-test; P = 0.03), *Halospirulina* spp. (Kruskal-Wallis test; X^2 = 8.35; df = 2; P = 0.01; post-hoc Nemenyi-test; P = 0.01), and *Burkholderia* spp. (Kruskal-Wallis test; X^2 = 8.7; df = 2; P = 0.01; post-hoc Nemenyi-test; P = 0.01). The microbiome of adults in the control and high temperature treatment also differed significantly in the relative abundance of the less abundant genera (with a relative abundance lower than 3%) (Kruskal-Wallis test; X^2 = 7.3; df = 2; P = 0.03; post-hoc Nemenyi-test; P = 0.02). The differences among

temperature treatments were not intuitive due to the low replication and high variability within the treatments (Table 4.5).

Alpha diversity: Diversity within the samples

The diversity of the microbiome of *T. granarium* adults differed significantly among temperature treatments for some of the diversity measures (Table 4.6). The Shannon index (Kruskal-Wallis test; $X^2 = 7.4$; $df = 2$; $P = 0.02$; post-hoc Nemenyi-test; $P = 0.02$) and observed number of OTUs (Kruskal-Wallis test; $X^2 = 7.9$; $df = 2$; $P = 0.02$; post-hoc Nemenyi-test; $P = 0.01$) were significantly higher in the samples exposed to high temperature treatment compared to the control. Conversely, Faith's phylogenetic diversity (Kruskal-Wallis test; $X^2 = 5.7$; $df = 2$; $P > 0.05$) and Chao1 (richness) (Kruskal-Wallis test; $X^2 = 2.7$; $df = 2$; $P > 0.05$) did not show any significant differences among the temperature treatments.

Beta diversity: Diversity between the samples

The microbial community composition of *T. granarium* adults exposed to different temperatures was significantly different only when the composition of the microbiome is considered. There were significant differences in the microbiome composition among the samples (Fig. 4.4A; Adonis; $F_{2, 14} = 1.53$; $R^2 = 0.179$; $P = 0.01$), 18% of the variation could be explained by temperature. Conversely, when considering relative abundances of the bacterial taxa, the microbiomes of adults did not significantly differ among the temperatures (Fig. 4.4B; Adonis; $F_{2, 14} = 1.71$; $R^2 = 0.196$; $P > 0.05$), which explained 19.6% of the observed variation. Principal coordinate analysis considering the abundance

of the bacteria (Fig. 4.4B) showed two clusters of samples on the PC1 (91.51% difference) but these clusters were unrelated to the different temperature treatments.

Discussion

The microbiomes of T. variabile *and* T. granarium

The microbiomes of *T. variabile* and *T. granarium* share a common core microbiome composed of *Spiroplasma*, *Pseudomonas*, and *Halospirulina*. Within the core microbiome, bacteria from the genus *Spiroplasma* spp. are the only ones known to be common facultative symbionts of insects (present in about 5 - 10% of all insect species) (Herren *et al.*, 2013). Bacteria from the genus *Spiroplasma* can manipulate insects' reproduction (Herren *et al.*, 2013). Species from the genus *Pseudomonas* are commonly soil- and plant-associated, but have been previously reported in insects from a wide range of habitats (Robinson *et al.*, 2010, Reid *et al.*, 2011, Welch *et al.*, 2015). In some studies, *Pseudomonas* spp. have been found to be pathogenic (Chen *et al.*, 2014, Vodovar *et al.*, 2006, Opota *et al.*, 2011), beneficial (Ceja-Navarro *et al.*, 2015), or to be acquired from the environment without any known functions in the insect (Welch *et al.*, 2015). The genus *Halospirulina* from the phylum Cyanobacteria is found mainly in hypersaline water, but it is not commonly found in insects (Nubel *et al.*, 2000). Similarly, members of the Solibacteraceae in *T. variabile* are mainly found in acidic soils (Landesman *et al.*, 2014).

Two members of the core microbiome, *Halospirulina* spp. and the family Solibacteraceae, have been rarely reported in insects. Although future studies may identify more cases of these bacteria from insects, these results might potentially be due

to the acquisition of bacteria from the environment or to PCR and sequencing artifacts (Kembel *et al.*, 2012, Poretsky *et al.*, 2014). Sequences from environmental bacteria can be introduced into the digestive system with the diet (Yun *et al.*, 2014). However, it is unlikely that the dry, grain-based diet used in this study would contain high quantities of soil and water-associated bacteria (Nubel *et al.*, 2000, Landesman *et al.*, 2014). When considering the PCR and sequencing bias, the short reads of the 16S rRNA gene can lead to sequencing errors and has a limited resolution among closely related bacterial species (Poretsky *et al.*, 2014). Denoising and chimera checking are not able to eliminate all biases and erroneous sequences, as well as misidentification errors and limitations associated with the databases used to identify the sequences (Kembel *et al.*, 2012, Poretsky *et al.*, 2014, Di Bella *et al.*, 2013). During sample preparation, there can also be the introduction of contaminating DNA from molecular reagents (Poretsky *et al.*, 2014, Salter *et al.*, 2014). Contaminating DNA from reagents would match sequences from water- and soil- associated bacteria mainly (Poretsky *et al.*, 2014). The presence of contaminating sequences becomes important in samples with a low microbial biomass (Salter *et al.*, 2014). For future studies, it is recommended to sequence an additional blank sample containing a known "synthetic community" to control for sequencing bias and contamination (Poretsky *et al.*, 2014, Salter *et al.*, 2014).

The microbiomes of *T. variabile* and *T. granarium* differ mainly in the relative abundances of their bacterial genera, which greatly affects the diversity of the samples (Table 4.1). The microbiome of *T. granarium* is dominated by *Spiroplasma* spp. (99.6% relative abundance), which is the main difference with the microbiome of *T. variabile* that does not have a dominant genus (Hughes *et al.*, 2001, Colwell, 2009). In *T. granarium*,

the high relative abundance of *Spiroplasma* spp. decreased the evenness in the community and the probability of finding rare species present in low relative abundances (fewer observed OTUs and lower phylogenetic diversity) (Table 4.2) (Hughes *et al.*, 2001, Colwell, 2009). However, the estimated true richness (Chao1) of the microbiome is not different between the two insect species, and if more sampling were to be done the number of observed OTUs are estimated to not differ between the hosts (Chao, 1984). These results are further supported by the beta-diversity analysis (Fig. 4.2), where the differences between the microbiomes of *T. variabile* and *T. granarium* are more explained by the abundances (73%) rather than presence/absence of taxa (26%).

Interspecific variation in the relative abundance of *Spiroplasma* spp. suggests a key host/microbe association with *T. granarium* (Meriweather *et al.*, 2013). Microbial communities are often more similar among closely related than in more distantly related hosts (Moran & Sloan, 2015). However, the microbiomes of *T. granarium* and *T. variabile* differed in the abundance of *Spiroplasma* even though both species were reared on the same diet and under the same conditions. Therefore, genetic, immunologic or behavioural differences between the two insect species might allow a higher colonization, persistence and transmission of *Spiroplasma* spp. in *T. granarium*, (Chandler *et al.*, 2011, Franzenburg *et al.*, 2013). For example, adults of *T. variabile* both fly and feed, whereas adults of *T. granarium* do neither (Loschiavo, 1960, Musa & Dike, 2009). Previous studies in insects have shown that the presence of facultative symbionts (i.e., *Spiroplasma*, *Wolbachia* and *Cardinium*) vary among members of the same or related host species sharing the same ecological niche, although they can be transferred within and among species (Moran *et al.*, 2008). *Wolbachia*, a facultative symbiont, has been

detected in the stored-product pest *Tribolium confusum*, but it is absent in its congeneric *T. castaneum*, although both species commonly co-occur (Li *et al.*, 2015)

Spiroplasma can be mutualists, incidental commensals, or pathogenic depending on the strain (Anbutsu & Fukatsu, 2011). These bacteria can increase in frequency in the population by manipulating host reproduction and (or) by improving host fitness (Herren *et al.*, 2013). In insects, they can cause cytoplasmic incompatibility, manipulation of sex ratios, male killing (Anbutsu *et al.*, 2008, Anbutsu & Fukatsu, 2011, Herren *et al.*, 2013), and they can confer resistance to parasitic wasps (Xie *et al.*, 2010) and nematodes (Jaenike *et al.*, 2010). Further studies are needed to determine the importance of *Spiroplasma* infection in *T. granarium*; e.g., the testing of multiple populations (wild and laboratory colonies), characterization of the *Spiroplasma* species and strain, and the assessment of its effects on *Trogoderma* hosts.

Effects of life stage on the microbial community of T. granarium

The microbiome of *T. granarium* and the relative abundance of *Spiroplasma* changed significantly with the life stage. Differences among life stages are mainly due to differences in the abundance of *Spiroplasma*; a high relative abundance of this bacterium means low diversity within the samples (Tables 4.3, 4.4) (Hughes *et al.*, 2001). The weighted UniFrac analysis showed a separation among samples with a relative abundance of *Spiroplasma* lower than 25% from the other samples with higher abundances (Fig. 4.3B). All life stages of *T. granarium* contained *Spiroplasma* in their microbiome in relative abundances > 1% except for non-diapausing non-acclimated larvae. This life stage also had the highest diversity, evenness, and richness, and a higher abundance of

rare genera (Tables 4.3, 4.4). This result is expected because of the active feeding state of the non-diapausing non-acclimated larvae, whereas, the other life stages do not feed (adults, pupae and eggs) or feed only occasionally (diapausing larvae, diapausing-acclimated larvae) (Burges, 1959a). The non-diapausing non-acclimated larvae have higher abundances of genera that are commonly associated with the digestive tract of insects or acquired from the environment; i.e., *Pseudomonas* spp. (Engel & Moran, 2013, Bansal *et al.*, 2014), *Lactococcus* spp.(Tagliavia *et al.*, 2014), *Anoxybacillus* spp. (Rogers & Backus, 2014), *Staphylococcus* (Engel & Moran, 2013), and *Streptococcus* spp.(Martin & Mundt, 1972). These bacteria might have been present in the diet contained in the larval intestine, being functionally specialized to facilitate digestion in the larvae (Crotti *et al.*, 2010, Engel & Moran, 2013) or persisting in higher numbers in the intestine, because of favourable internal physicochemical conditions and the availability of nutrients (Engel & Moran, 2013). For example, a complex protein system with at least six proteinases has been detected in the midgut of *T. granarium* larvae, whereas in adults no proteolytic or amylolityc activities were detected, meaning that adults are unable to digest any food, which is consistent with the fact that adults do not eat (Hosseininaveh *et al.*, 2007).

Spiroplasma bacteria were present in higher abundances in non-feeding stages; i.e., adults and pupae (Table 4.3). The lower diversity and richness of the microbiome of these stages might due to the moulting of the foregut lining, which disrupts or eliminates bacterial populations attached to it (Engel & Moran, 2013). In adults and pupae of *T. granarium*, these bacterial populations cannot be fully recovered due to the lack of feeding (Moran *et al.*, 2008). They could be partially recovered in diapausing and diapausing-acclimated

larvae by occasional feeding, but could be lost by the frequent molting of these stages (Burges, 1959a). Therefore, the microbial communities of diapausing stages can also be affected by the time of the last molt or the time of last feeding. The presence of *Spiroplasma* spp. in all life stages, even after molting, is not surprising because it can persist in the hemolymph (Herren *et al.*, 2013). Future studies using quantitative PCR would allow quantifying the effect of life stage on the abundances of *Spiroplasma*.

The dominance of *Spiroplasma* in eggs and its high relative abundance in adults support the hypothesis of its vertical transmission in *T. granarium* (Herren *et al.*, 2013). In vertically transmitted symbioses, the symbiotic association is permanent in the insect and present in all of the insect's life stages (Bright & Bulgheresi, 2010). The higher relative abundance of *Spiroplasma* in adults and pupae agrees with previous studies on the *Spiroplasma-Drosophila* relationship; i.e., the number of DNA copies of *Spiroplasma* increases in the pupal stage, after adult emergence it keeps increasing with adult age, and during the late phases of egg formation (Bright & Bulgheresi, 2010, Herren *et al.*, 2013, Anbutsu & Fukatsu, 2011). Depending on the species, commensal and pathogenic *Spiroplasma* bacteria can be transmitted horizontally, but endosymbiotic species are unable to survive outside the host and have a transovarial vertical transmission through the eggs (Herren *et al.*, 2013). In the eggs, the presence of sequences from other bacteria that are not known to be transmitted transovarially could be due to contaminating DNA or egg smearing by the adults (contamination with feces during oviposition) (Bright & Bulgheresi, 2010, Dematheis *et al.*, 2012, Funkhouser & Bordenstein, 2013). Although the eggs were sterilized, the low microbial load inside the sample enhances the likelihood of detecting contaminating DNA that might not have been completely removed during

washing or which might have been present in the reagents (Kembel *et al.*, 2012, Dematheis *et al.*, 2012, Poretsky *et al.*, 2014). Previous studies on eggs from other groups of insects have also reported the presence of multiple genera of bacteria (Hail *et al.*, 2012, Dematheis *et al.*, 2012, Palavesam *et al.*, 2012). Future studies should consider the stability of *Spiroplasma* infection, its transmission and prevalence in the population, and its role in host biology and reproduction.

Effects of temperature on the microbial community of T. granarium

The microbiome of *T. granarium* adults exposed to extreme temperatures did not differ despite a lower relative abundance of *Spiroplasma* in insects at high temperature. When compared to other temperatures, insects that were exposed to high temperatures had lower relative abundances of *Spiroplasma* spp., higher number of OTUs, and increased values of evenness and Shannon index (Tables 4.5, 4.6). However, there were no differences in the true estimated richness of the microbiome or in phylogenetic diversity. These results are confirmed by the beta diversity analysis, which showed two clusters in the PC1 unrelated to the temperature treatments (Fig. 4.4B).

Because the maximum temperature for optimal growth of *Spiroplasma* bacteria is 26 - 30°C (Anbutsu & Fukatsu, 2011), it was expected that the suboptimal temperatures used in this study would reduce the quantity of this bacteria in *T. granarium*. In previous studies in *Drosophila* hosts, temperatures lower than 18^{o}C supressed the infection density and vertical transmission of *Spiroplasma*, while temperatures higher than 28^{o}C mainly affected its vertical transmission (Anbutsu *et al.*, 2008, Anbutsu & Fukatsu, 2011). Similarly, in the sawtoothed grain beetle *Oryzaephilus surinamensis* (Coleoptera:

Silvanidae), the obligate symbionts present in the insect's mycetomes (organs specialized to host symbionts) can be eliminated by exposing the insect to low or high temperatures (Buchner, 1965). Although the relative abundance of *Spiroplasma* decreased in the high temperature treatment, the metagenomic approach used in this study considers all the sequences present in the sample, which represents active and inactive (or dead) bacteria (Reid *et al.*, 2011, Di Bella *et al.*, 2013). Therefore, in future studies the application of quantitative PCR and proteomics approaches should be considered to assess the effect of temperature on the survival of *Spiroplasma*.

Despite the economic importance of *T. granarium* as a pest of stored-products, to my knowledge this is the first study on the microbiome of this insect. The microbiome of *T. granarium* has a high relative abundance of *Spiroplasma* bacteria and the vertical transmission of these bacteria is supported by its presence in eggs. These bacteria might represent a key host/symbiont interaction that can be used to design and improve control methods against this pest (Zindel *et al.*, 2011, Meriweather *et al.*, 2013). *Trogoderma granarium* has a high tolerance to insecticides and to extreme temperatures that could be associated with its microbiome (Zindel *et al.*, 2011, Wilches *et al.*, 2016). The manipulation of the microbiome could increase the susceptibility of *T. granarium* to the current methods of control or be used in the development of new methods (Sinkins *et al.*, 1997, Zindel *et al.*, 2011). For example, the use of cytoplasmic incompatibility to reduce the population of the insect, paratransgenesis (i.e., the expression of a gene in the symbiont that is detrimental to the insect), or the use of symbionts as carriers of entomopathogenic viruses (Zindel *et al.*, 2011, Knight & Witzgall, 2013, Minard *et al.*, 2014), are worthy of future study. Further research is also needed to identify the effects

and dynamics of *Spiroplasma* spp. in *T. granarium* and to evaluate the potential for the use of these bacteria as a method of control.

Conclusions

1. The core microbiomes of *T. granarium* and *T. variabile* are similar in composition, but they differ in the relative abundance of *Spiroplasma* bacteria. *Spiroplasma* is present in a relative abundance of more than 90% in *T. granarium* whereas the relative abundances in *T. variabile* are lower than 3%.
2. The microbiome of *T. granarium* differs among life stages. *Spiroplasma* bacteria are present in all life stages including eggs, which suggests a vertical transmission of these bacteria from mother to eggs.
3. No effect of low temperature was detected on the microbiome of *T. granarium* adults. An effect of high temperature was detected in reducing the relative abundances of *Spiroplasma* spp. and increasing the diversity of the microbiome.
4. This study was based on one laboratory population each of *T. granarium* and *T. variabile*. Future studies should survey multiple laboratory and wild populations for the presence of *Spiroplasma* spp., its effects on fitness, its prevalence, and the stability of the transmission in *T. granarium.*
5. Further research should consider the use of quantitative PCR and proteomics to assess the effects of *Spiroplasma* on *T. granarium* and the effects of temperature on the microbiome.
6. The study of the microbiome of *T. granarium* should be considered to develop new methods of control and to improve the efficiency of current methods.

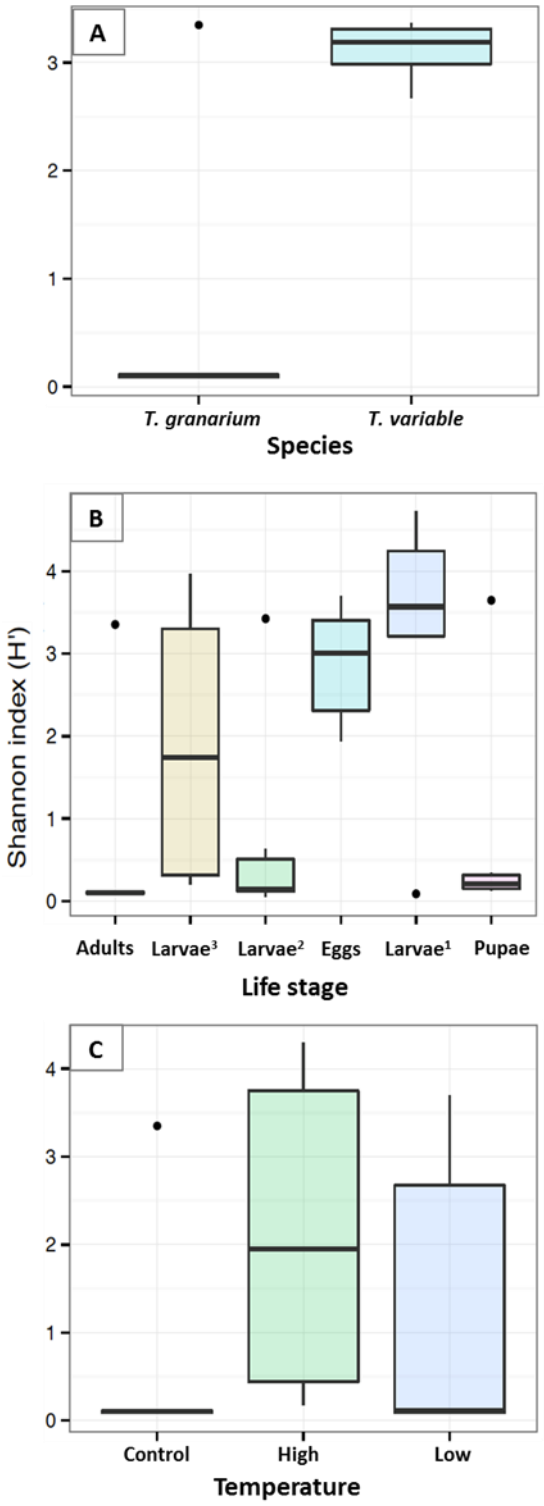


Figure 4.1 Alpha diversity measured as Shannon index (H') for each of the treatments in A) Study 1 B) Study 2 C) Study 3.

[1]Non-diapausing non-acclimated larvae; [2]Diapausing larvae; [3]Diapausing-acclimated larvae

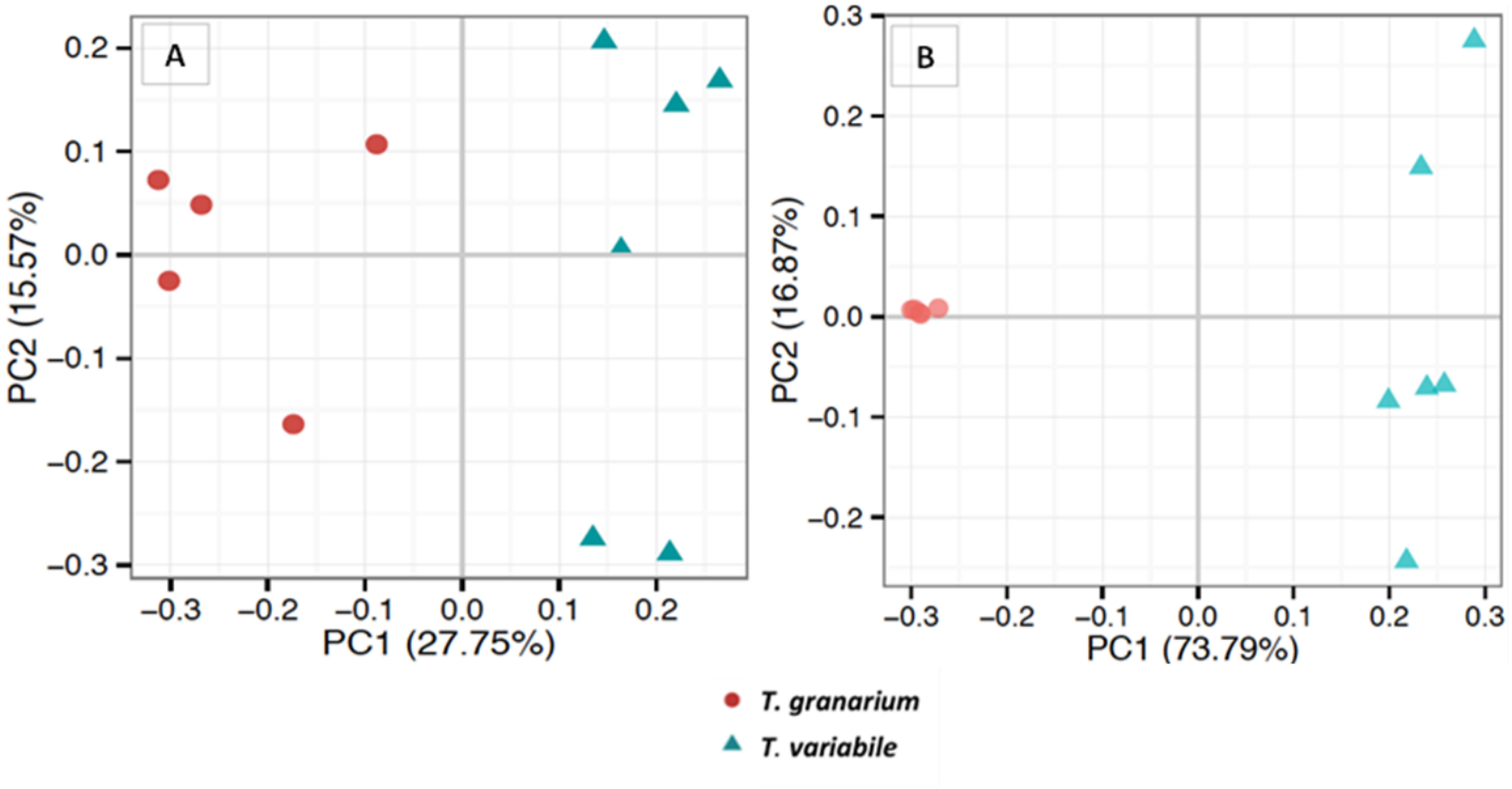
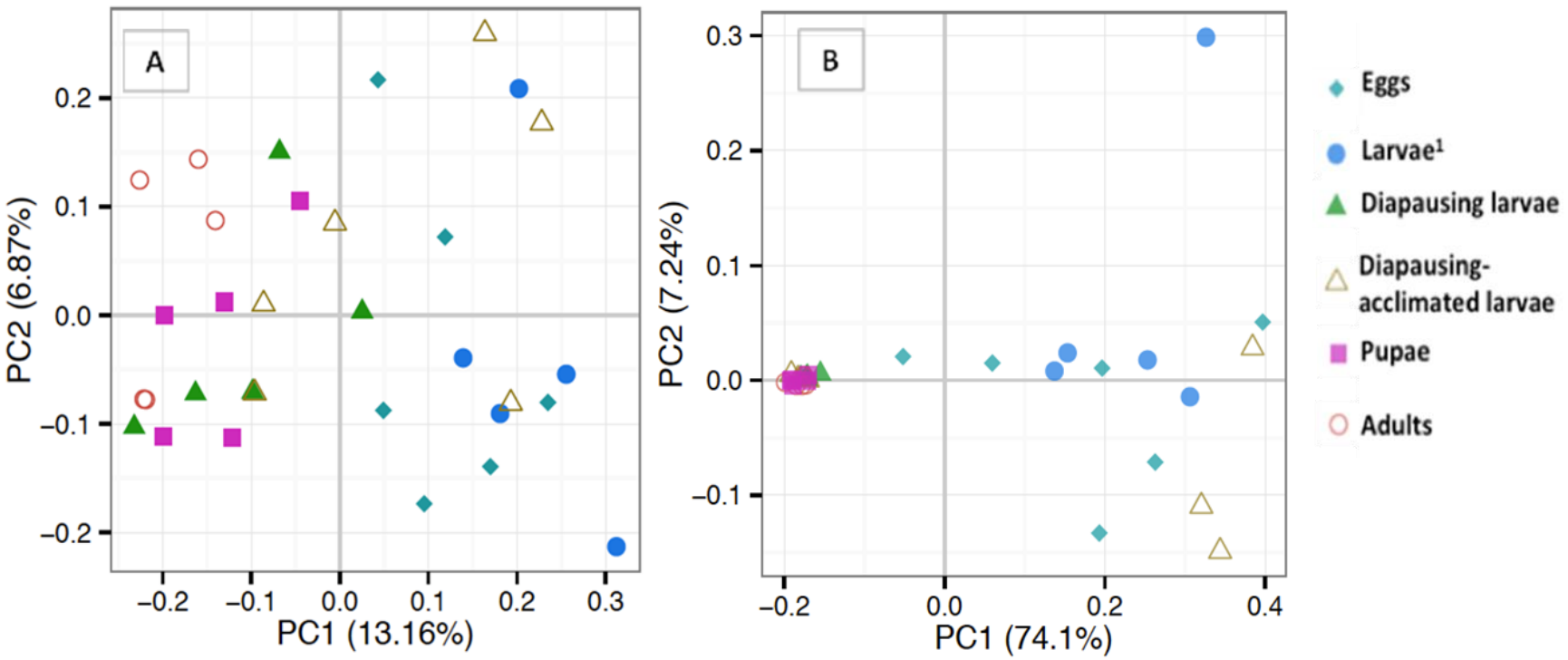


Figure 4.2 Study 1. Principal coordinates analyses showing differences among samples of *T. granarium* (*n* = 5) and *T. variabile* (*n* = 6) based on differences in: **A)** the presence/absence of taxa that comprise the microbiome. **B)** the relative abundance of taxa that comprise the microbiome (Note that 5 samples from *T. granarium* are clustered together in the PC1 and PC2)



[1]Non-diapausing non-acclimated larvae

Figure 4.3 Study 2. Principal coordinates analyses showing differences among samples of different life stages of *T. granarium*: Eggs (*n* = 6), Larvae [1](*n* = 5), diapausing larvae (*n* = 5), diapausing-acclimated larvae (*n* = 6), pupae (*n* = 5), and adults (*n* = 5). The analysis was based on differences in: **A)** the presence/absence of taxa that comprise the microbiome. **B)** the relative abundance of taxa that comprise the microbiome (18 samples from different life stages are clustered closely together close to the PC2 axis).

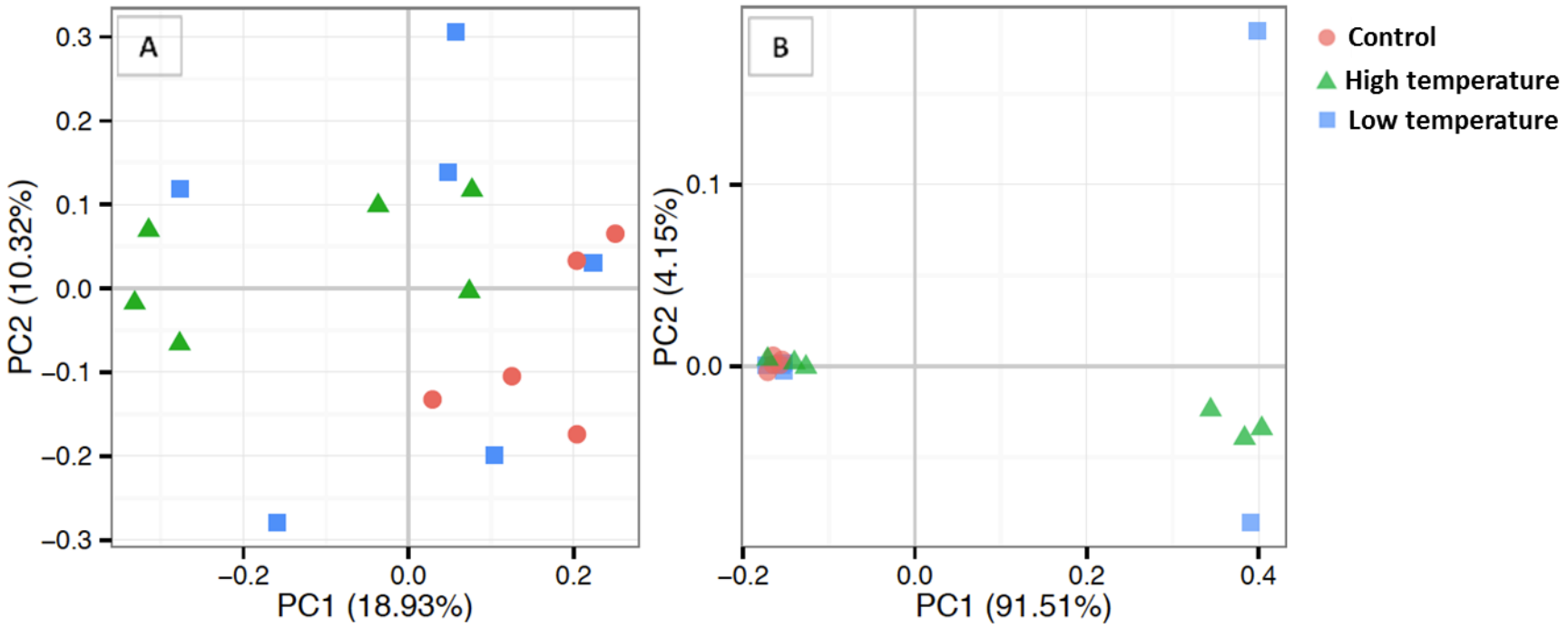


Figure 4.4 Study 3. Principal coordinates analyses showing differences among samples of *T. granarium* exposed to low (−15°C, *n* = 6), control (30°C, *n* = 5) and high (50°C, *n* = 6) temperatures based on differences in: **A)** the presence/absence of taxa that comprise the microbiome. **B)** the relative abundance of taxa that comprise the microbiome (Note that 12 samples from different temperatures are clustered closely together on the PC2 axis).

Table 4.1 Study 1. Mean relative abundances (%) of the most abundant bacterial genera (with relative abundance > 3% in at least two samples) of the microbiome of *T. granarium* (*n* = 5) and *T. variabile* (*n* = 6). The asterisks indicate significant differences between the two species (Wilcoxon rank sum test; $P < 0.05$). The members of the core microbiome with high relative abundances are in bold.

	Relative abundance (%) ± SE	
Bacterial genera	***T. granarium***	***T. variabile***
Spiroplasma *	99.651 ± 0.035	1.423 ± 0.689
Halospirulina *	0.078 ± 0.008	30.321 ± 7.355
Pseudomonas *	0.067 ± 0.011	16.172 ± 5.069
Caulobacter	0.027 ± 0.009	10.365 ± 2.521
Ralstonia	0.031 ± 0.008	7.786 ± 4.038
Delftia	0.012 ± 0.004	4.210 ± 1.538
Staphylococcus	0.050 ± 0.044	5.491 ± 3.585
Other genera*	0.064 ± 0.053	17.875 ± 1.823

Table 4.2 Study 1. Mean alpha diversity of the microbiome of *T. granarium* (*n* = 5) and *T. variabile* (*n* = 6) measured as Shannon index, Chao1, Faith's phylogenetic diversity and observed OTUs. The asterisks indicate significant differences between the two species (Wilcoxon rank sum test; $P < 0.05$).

	Mean ± SE	
Alpha-diversity measure	***T. granarium***	***T. variabile***
Shannon index*	0.096 ± 0.005	3.11 ± 0.11
Chao1	90.30 ± 9.46	113.32 ± 2.62
Faith's phylogenetic diversity*	3.18 ± 0.11	5.16 ± 0.28
Observed OTUs*	31.10 ± 1.13	63.80 ±3.40

Table 4.3 Study 2.Mean number of sequences per life stage and mean relative abundances (%) of the most abundant bacterial genera (with a mean relative abundance higher than 3%, and present in at least two samples) of the microbiome of different life stages of *T. granarium*. Means within rows followed by different letters are significantly different (Kruskall-Wallis test; $P < 0.05$; Nemenyi-tests; $P < 0.05$). The members of the core microbiome with high relative abundances are in bold.

	Relative abundance (%) ± SE					
Bacterial genera	**Eggs (*n* = 6)**	**Larvae[1] (*n* = 5)**	**Diapausing Larvae (*n* = 5)**	**Diapausing-acclimated Larvae (*n* = 6)**	**Pupae (*n* = 5)**	**Adult (*n* = 5)**
Spiroplasma	25.9 ± 11.7 ***bc***	0.7 ± 0.2***c*** [2]	98.1 ± 1.2 ***abc*** [2]	49.4 ± 21.8 ***bc***	99.34 ± 0.15 ***ab***	99.65 ± 0.035 ***a***
Halospirulina	12.8 ± 5.3 ***a***	8.5 ± 2.0 ***a***	0.3 ± 0.1 ***ab***	16.6 ± 7.3 ***a***	0.16 ± 0.034 ***ab***	0.078 ± 0.0079 ***b***
Pseudomonas	8.4 ± 1.7 ***a***	8.4 ± 4.3 ***a***	0.1 ± 0.05 ***ab***	10.53 ± 5.45 ***ab***	0.10 ± 0.027 ***ab***	0.067 ± 0.011***b***
Caulobacter	0.009 ± 0.002 ***a***	2.3 ± 0.6 ***b***	0.06 ± 0.02 ***ab***	1.8 ± 1.12 ***ab***	0.034 ± 0.008 ***ab***	0.027 ± 0.0039 ***ab***
Ralstonia	2.4 ± 2.4	6.1 ± 3.9	0.009 ± 0.002	0.009 ± 0.0023	0.024 ± 0.008	0.03 ± 0.008
Delftia	1.3 ± 0.5	1.4 ± 0.7	0.07 ± 0.03	0.05 ± 0.03	0.021 ± 0.012	0.012 ± 0.0045
Streptococcus	0.3 ± 0.3 ***a***	19.3 ± 7.2 ***b***	0.28 ± 0.27 ***ab***	0.012 ± 0.004 ***ab***	0.012 ± 0.002 ***a***	0.016 ± 0.001 ***ab***
Staphylococcus	3.3 ± 2.6	5.2 ± 3.9	0.04 ± 0.03	4.3 ± 4.2	0.0084 ± 0.0021	0.0086 ± 0.00096
Bacillus	5.5 ± 2.5	0.3 ± 0.3	0.006 ± 0.001	1.08 ± 1.07	0.02 ± 0.01	0.005 ± 0.0013
Geobacillus	8.6 ± 7.1	2.5 ± 0.8	0.19 ± 0.18	0.008 ± 0.002	0.009 ± 0.001	0.008 ± 0.0009
Anoxybacillus	0.9 ± 0.6	8.2 ± 3.5	0.32 ± 0.31	0.035 ± 0.026	0.008 ± 0.001	0.01 ± 0.002
Lactococcus	0.4 ± 0.4 ***ab***	8.6 ± 4.4 ***a***	0.079 ± 0.076 ***ab***	0.0035 ±0.0014 ***b***	0.004 ± 0.0009 ***ab***	0.0055 ± 0.0016 ***ab***
Citrobacter	0.53 ± 0.52	0.4 ± 0.4	0.037 ± 0.034	2.72 ± 1.7	0.009 ± 0.007	0.013 ± 0.007
Other genera	30.1 ± 11.6 ***a***	28.0 ± 12.0 ***a***	0.35 ± 0.11 ***ab***	13.4 ± 6.8 ***a***	0.24 ± 0.11 ***ab***	0.067 ± 0.0065 ***b***
Mean number of sequences ± SE	19586 ± 135.7 ***ab***	17880 ± 890.7 ***b***	19966 ± 9.84 ***a***	18588 ± 554 ***b***	19700 ± 89.6 ***ab***	19943 ± 7.83 ***a***

[1]Non-diapausing non-acclimated larvae;[2] $P = 0.057$ for non-diapausing non-acclimated larvae and diapausing larvae comparison

Table 4.4 Study 2. Mean alpha diversity of the microbiome of different life stages of *T. granarium* measured as Shannon index, Chao1, Faith's phylogenetic diversity and observed OTUs. Means (±SE) within rows followed by different letters are significantly different (Kruskall-Wallis test; $P < 0.05$) (Nemenyi-tests; $P < 0.05$).

	Mean ± SE					
Alpha-diversity measure	**Eggs (*n* = 6)**	**Larvae[1] (*n* = 5)**	**Larvae[2] (*n* = 5)**	**Larvae[3] (*n* = 6)**	**Pupae (*n* = 5)**	**Adults (*n* = 5)**
Shannon index	2.88 ± 0.294***a***	3.89 ± 0.30***a***	0.22 ± 0.105***b***	1.88 ± 0.712 ***a***	0.21 ± 0.04***ab***	0.095 ± 0.0055***b***
Chao 1	218.2 ± 19.1***a***	270.2 ± 51.5***a***	121.3 ± 23.6***ab***	130.6 ± 14.2***ab***	108.3 ± 11.3***ab***	100.7 ± 7.58***b***
Faith's phylogenetic diversity	6.03 ± 0.46***a***	7.7 ± 1.1***a***[4]	4.5 ± 0.5***ab***	4.9 ± 0.35***ab***	4.02 ± 0.14***ab***[4]	3.56 ± 0.19***b***
Observed OTUs	79.2 ± 7.4***ab***	110.4 ± 18.2***a***	43.3 ± 7.1***bc***	59.6 ± 7.6***abc***	43.5 ± 3.8***abc***	36.5 ± 1.5***c***

[1]Non-diapausing non-acclimated larvae; [2]Diapausing larvae; [3]Diapausing-acclimated larvae; [4] $P = 0.053$ for comparison between non-diapausing non-acclimated larvae and pupae.

Table 4.5 Study 3. Mean relative abundances (%) of the most abundant bacterial genera (with a mean relative abundance higher than 3%, and present in at least two samples) of the microbiome of adults of *T. granarium* exposed to −15°C (42 h) (*n* = 6), 30°C, and 50°C (4 h) temperatures. Means within rows followed by different letters are significantly different (Kruskall-Wallis test; $P < 0.05$; Nemenyi-tests; $P < 0.05$). The members of the core microbiome with high relative abundances are in bold.

	Relative abundance (%) ± SE		
Bacterial genera	**−15°C (*n* = 6)**	**30°C (*n* = 5)**	**50°C (*n* = 6)**
Spiroplasma	66.7 ± 20.8***ab***	99.65 ± 0.035***a***	50.8 ± 20.7***b***
Halospirulina	10.9 ± 7.2***ab***	0.078 ± 0.0079***a***	14.1 ± 6.2***b***
Pseudomonas	7.02 ± 4.4	0.067 ± 0.011	8.2 ± 3.3
Caulobacter	0.13 ± 0.127***a***	0.027 ± 0.004***ab***	5.3 ± 2.8***b***
Ralstonia	3.1 ± 3.0	0.03 ± 0.008	0.90 ± 0.88
Delftia	0.013 ± 0.006	0.012 ± 0.0045	0.08 ± 0.03
Burkholderia	0.007 ± 0.003***ab***	0.002 ± 0.0003***a***	4.0 ± 2.9***b***
Propionibacterium	3.0 ± 1.9	0.0082 ± 0.0018	0.44 ± 0.36
Other genera	9.2 ± 7.0 ***ab***	0.12 ± 0.012***b***	16.2 ± 7.1***a***

Table 4.6 Study 3. Mean alpha diversity of the microbiome measured as Shannon index, Chao1, Faith's phylogenetic diversity, and observed OTUs of the microbiome of adults of *T. granarium* exposed to low (−15°C), control (30°C), and high (50°C) temperatures. Means within rows followed by different letters are significantly different (Kruskall-Wallis test; $P < 0.05$) between temperatures (Nemenyi-tests; $P < 0.05$).

	Mean ± SE		
Alpha-diversity measure	**Low (*n* = 6)**	**Control (*n* = 5)**	**High (*n* = 6)**
Shannon index	1.27 ± 0.74***ab***	0.093 ± 0.006***b***	2.11 ± 0.786***a***
Chao 1	118.97 ± 19.62	90.58 ± 11.24	144.2 ± 35.01
Faith's phylogenetic diversity	4.13 ± 0.49	3.31 ± 0.17	5.19 ± 0.65
Observed OTUs	46.6 ± 9.2ab	31.6 ± 1.14b	65.3 ± 12.1a

CHAPTER 5: GENERAL DISCUSSION

Summary

Trogoderma granarium, khapra beetle, is among the most important invasive species and pests of stored agricultural products (Eliopoulos, 2013). This pest is considered a quarantine insect for Canada, United States and Australia (OEPP/EPPO, 2013, OEPP/EPPO, 2015). If introduced into these countries, it could cause high economic losses (Eliopoulos, 2013). The high importance of *T. granarium* worldwide lies in the damage it can cause to commodities and in the resilience of its diapausing larvae, which makes it difficult to control (Lindgren & Vincent, 1959). The larvae of *T. granarium* can enter a state of facultative diapause; i.e., a developmental arrest that is triggered by external cues (Tauber *et al.*, 1986, Koštál, 2006). The diapause state increases larval tolerance to high doses of insecticides and to extreme conditions (Lindgren & Vincent, 1959, Bell *et al.*, 1984).

In Chapter 2, I investigated the induction of diapause in conditions of high density of larvae. The results agreed with previous studies in that the percentage of larvae in diapause increased with higher density of larvae and can be induced at high temperature (Burges, 1963, Nair & Desai, 1972). However, variability in the percentage of larvae that go into diapause seems to depend on the genetic background of the population and rearing conditions (Tauber & Tauber, 1976, Masaki, 2002). Larvae were reared at high densities to obtain diapausing individuals for all the experiments in this thesis.

The use of extreme temperatures might be a feasible alternative to methyl bromide for the control of *T. granarium* in pre-shipment and quarantine conditions. Fumigation with

methyl bromide has been for many years the most-used method to control *T. granarium*, but this fumigant is an ozone-depleting substance. Therefore, the Montreal Protocol has banned the use of methyl bromide on a global basis with some exceptions (e.g., quarantine uses) (Lindgren & Vincent, 1959, Linnie, 1994, MBTOC, 2014, Fields & White, 2002). Currently, there is an increasing need to develop new methods of control for *T. granarium*, e.g., extreme temperatures (Fields & White, 2002, Eliopoulos *et al.*, 2011, Fields *et al.*, 2012). The adoption of extreme temperatures to control *T. granarium* has been hampered by an inability to increase or decrease temperatures economically and quickly, and the absence of knowledge of the combination of temperature and period of exposure needed for control (Strang, 1992, Bergh *et al.*, 2006).

The assessment of the time-temperature combination necessary to control *T. granarium* should consider the factors affecting temperature tolerance; i.e., life history and biology of the species, life stage, diapause, relative humidity, and acclimation (previous exposure to high/low temperature) (Salt, 1958, Evans, 1986, Lee, 1991, Fields, 1992, Fields, 2001, Beckett *et al.*, 2007). Chapters 2 and 3 aimed to determine the temperature-time combinations needed to control all life stages of *T. granarium* with low and high temperatures, respectively. These chapters also assessed the effect of life stage, diapause, and acclimation in temperature tolerance. This objective was achieved in a two-step-process: 1) exposing different life stages to cold and high temperatures to determine the most cold- and heat-tolerant life stages, and 2) determining the time necessary to kill 99% of the population at different high and low temperatures. The most cold- and heat-tolerant life stages were determined to be diapausing-cold acclimated larvae and diapausing larvae, respectively. These results confirmed the hypotheses of diapause increasing

tolerance at low and high temperatures, and acclimation being important to increase tolerance at low temperatures. The combination of acclimation and diapause increased cold-tolerance more than either one of them independently (Abdelghany *et al.*, 2015). There is little evidence suggesting that heat acclimation greatly increases the heat-tolerance at the temperatures used for pest control (50-60^{o}C) (Evans, 1986), but this factor still needs to be assessed in *T. granarium*. Based on these results, the control of *T. granarium* with low temperatures should target diapausing-acclimated larvae, whereas use of high temperatures should target the diapausing larvae.

Trogoderma granarium has a high tolerance to low (from 0 to −15°C) and high temperatures (from 45 to 60°C), which is unusual for stored-product pests (Howe, 1965, Fields, 1992). My results show that low-temperature control of diapausing-acclimated larvae (99% mortality) requires an exposure of 541, 340, 212 and 49 days at 0, −5, −10 and −15°C, respectively. Because of the long durations needed to control *T. granarium*, reducing temperatures to below the supercooling point of −25^{o}C may be the best way to control this insect with low temperature. At high temperatures, the control of diapausing larvae is achieved in 288 h at 45^{o}C, 6 h at 50^{o}C, 1.1 h at 55^{o}C, and 1 h at 60^{o}C.

The result of *T. granarium* being highly tolerant to high temperatures is expected due to its tropical origin, but the cold-tolerance of its diapausing-acclimated larvae is surprising and identifies it as one of the most cold- and heat-tolerant stored-product pests studied thus far. *Trogoderma granarium* is xerophilous (thrives in dry environments) and can survive feeding on food with a water content below 10% (Zhantiev, 2009). The mechanisms of injury caused by desiccation and low temperatures are similar in terms of osmotic stress and they elicit similar adaptations for survival (Convey, 1999, Everatt *et*

al., 2014). This insect might be pre-adapted to cold because of its adaptations to desiccation and high temperatures, and some unknown factors of its biology that provide high tolerance to extreme conditions (Sinclair *et al.*, 2013).

The microorganisms associated with insects (microbiome) can affect host tolerance to extreme temperatures and other methods of control (Douglas, 2007, Zindel *et al.*, 2011, Wernegreen, 2012). The members of the microbiome or symbionts can affect host fitness, resistance to natural enemies, resistance to insecticides, host reproduction, and tolerance to extreme temperatures (Zindel *et al.*, 2011, Wernegreen, 2012). The high tolerance of *T. granarium* to the methods of control and to extreme temperatures might be partially due to its microbiome, but this parameter has not been previously assessed. In Chapter 4, I investigated the microbiome of *T. granarium* using next-generation sequencing of the 16S rRNA gene. This chapter included 3 studies; Study 1 compared the microbiome of *T. granarium* and the congeneric *T. variable.* Study 2 and Study 3 assessed the effects of life stage and temperature on the microbiome of *T. granarium*. The core microbiomes of *T. granarium* and *T. variable* were similar in composition, but the relative abundances of *Spiroplasma* were significantly higher in *T. granarium* (higher than 90%) than in *T. variable* (lower than 3%). The microbiome of *T. granarium* was affected by life stage (feeding vs non-feeding life stages). The presence of *Spiroplasma* in *T. granarium* eggs suggests that it is vertically transmitted. High temperatures significantly reduced the relative abundance of *Spiroplasma*, but an effect of low temperatures on the microbiome of *T. granarium* was not detected. The failure to detect an effect could be due to cold temperatures not affecting the microbiome or the method used, which considers live and dead bacteria. For this reason, future studies investigating effect of temperatures on the

microbiome should use techniques that differentiate between dead and active bacteria; e.g., qPCR and proteomics. Further studies should be done to understand the association between *Spiroplasma* and *T. granarium.* Understanding the nature of the relationship *T. granarium*-microbiome and its effects may lead to the development of microbiome-based methods of control or to the improvement of the current ones.

Practical considerations and future directions

The application of temperature treatments to control *T. granarium* requires an acceptable cost and the development of procedures that avoid damage to products and facilities (Mason & Strait, 1998). The applicability of thermal treatments and the costs to reach the temperature and the length of time necessary for control will be affected by the size of the storage structure, and the type and quantity of the commodity (Mason & Strait, 1998, Fields & White, 2002). To use heat treatment, the grain can be treated using irradiation (infrared, microwave, and high frequency), dielectric heating, fluidized and sprouted beds (Mason & Strait, 1998). To apply cold treatment to grain products, the facilities and the product can be cooled to temperatures below -20^{o}C by methods such as turning of the grain to dissipate hot spots, ambient aeration in winter, chilled aeration (cool ambient air with refrigeration), and flash freezing with liquid nitrogen for high value items and herbs (Mason & Strait, 1998, Fields & White, 2002). In Canada and the northern United States, winter temperatures could be sufficiently cold to control *T. granarium* in structures using ambient aeration as long as the temperature outside stays below -15^{o}C for 50 days (LT_{99} for diapausing-acclimated larvae, Chapter 2, Table 2.2), or reaches −25°C for at least 1 day, which would cause instant freezing (Fields & White, 2002).

The methods to decrease or increase temperatures for thermal control require an energetic input and specialized equipment, but are still economically competitive with chemical-based pest management and methyl bromide fumigation (Mason & Strait, 1998, Fields & White, 2002, Hammond, 2015). Chilled aeration or freezing are most likely to be used to reach the low temperatures (−25°C for minutes) needed to control *T. granarium*. In the United States, the annual running costs for chilled aeration are estimated to be 2.20 USD per tonne (without considering initial cost of equipment) compared to 3.50 USD per tonne for phosphine fumigation and aeration (Fields, 1992, Mason & Strait, 1998, Rulon *et al.*, 1999, Fields & White, 2002). In Europe, in the summer, the price of heat treatment is often lower than the equivalent for methyl bromide operation. The costs of labour and consumables for heat treatment (energy for heating) of a 4000 m^3 flour mill is 4133 USD compared with 8206 USD for methyl bromide (Hammond, 2015). In addition, the quick decrease in temperature reached by chilled aeration can prevent acclimation in *T. granarium* and shorten the time to control larvae by more than 4-fold (Chapter 2, Table 2.1). The data on the mortality of *T. granarium* at different combinations of temperatures and duration of exposure can also be used in to develop of mathematical models to design case-specific treatment protocols and estimate costs (Fields, 1992, Abdelghany *et al.*, 2015). For the application of thermal treatments, mortality data can be modelled as a function of the time required for the facility or product to reach the lethal temperature, the exposure required to the lethal temperature, and the maximum/minimum temperature required for mortality (Subramanyam *et al.*, 2011). Models based on temperature can also be developed to predict the risk of future global spread and establishment of *T. granarium* (Paini & Yemshanov, 2012). According to my results, the diapausing-acclimated larvae of *T. granarium* might be able to survive in unheated grain storages in Canada as long as

the temperature does not drop below −15°C for more than 50 d (LT_{99} for diapausing-acclimated larvae, Chapter 2, Table 2.2). These results disagree with previous mathematical models that predict the risk of establishment of *T. granarium* in Canada (Ameen, 2012). These models may need to be reassessed, given new information on the thermal tolerance of cold-acclimated insects or insects in diapause, plus a general warming of temperatures in recent decades due to climate change (Estay *et al.*, 2009).

For quarantine uses, there are about 200 situations where low temperatures have been approved as an appropriate treatment (Fields & White, 2002); use of high temperatures is the only approved alternative to methyl bromide for the control of *T. granarium* (MBTOC, 2014). The heat treatment approved for *T. granarium* requires an exposure of 7 minutes at 65.5^{o}C and can only be used when specifically authorised by APHIS (Animal and Plant Health Inspection, United States) (MBTOC, 2014). For quarantine pests, the approval and application of alternative methods to methyl bromide have been hampered by the requirement to estimate the dose that causes 99.9968% of mortality (Probit 9) (Landolt *et al.*, 1984, Robertson *et al.*, 2007). The validity of the Probit 9 measure is questionable because its estimation is impractical (requires testing of high numbers of individuals), and the results are often inconsistent and highly variable. Furthermore, to reach the mortality of the Probit 9, high doses of the treatment need to be applied. This might be unnecessary for commodities with low levels of infestation and can negatively affect the product quality. Therefore, the estimation of the doses required for quarantine treatment should also consider the infestation level and the pest's probability of establishment (Follett & Neven, 2006, Robertson *et al.*, 2007). The probability of establishment depends on host availability, infestation rate of the commodity, probability

of a mating pair or mated female surviving the treatment, shipping conditions, shipment volume, and suitability of the climate (Follett & Neven, 2006). Alternatives to the Probit 9 that have been proposed for commodities that are rarely infested or have a low infestation rate; e.g., the maximum pest limit surviving in the commodity, and the alternative treatment efficacy approach that measures risk as the probability of a mating pair, gravid female, or parthenogenetic individual surviving in a commodity (Landolt *et al.*, 1984, Follett & McQuate, 2001). Thermal treatments cause mortality, developmental, and reproductive effects (Denlinger & Lee 1998, Denlinger & Yocum, 1998). An individual surviving the treatment might not be able to reproduce and establish a new population.

Future studies should assess the effects of the suggested temperature treatments for *T. granarium* on the quality and viability of the product to be treated. Low temperatures are less likely to reduce the quality of the product than high temperatures (Evans, 1986, Linnie, 1999). Temperatures as low as $-29^{o}C$ have little effect on the quality of products with low moisture content, but temperatures higher than $80^{o}C$ can lead to deleterious chemical and biochemical changes depending on the way the heat is delivered (Evans, 1986). Avoiding damage to the product requires studies specific to the product to determine the combination of temperature and duration exposure needed to achieve control, followed by post-treatment studies to assess product quality (e.g., for processed foods, preserved specimens, textiles) or viability (e.g., for bulk seeds) (Burges & Burrell, 1964, Evans, 1986, Fields, 1992, Fields & White, 2002). In practical terms, compared to cold treatment, the shorter times needed to control *T. granarium* at high temperatures makes the application of high temperatures more suitable for treatment of grain, but this

highly depends on the commodity to be treated (Burges & Burrell, 1964, Evans, 1986, Fields, 1992, Fields & White, 2002). Cold treatments can still be applied to sanitize packaging material, empty structures or production machinery once the product is removed (Hammond, 2015). Another alternative that has not been widely explored is the treatment of grain during transit using heating/cooling containers. During the transportation time heat/cold would have enough time to penetrate the commodity to control the pest (Hammond, 2015).

The application of temperature treatments together with other methods of control could shorten the time to mortality (Mason & Strait, 1998, Fields & White, 2002). *Trogoderma granarium* is among the more cold-tolerant stored-grain insect pests and the successful application of cold treatments on product would require shortening the time necessary to reach mortality. Chilled aeration requires a high initial investment and might be expensive and impractical to keep a constant temperature of -15^{o}C for the 50 d required to control *T. granarium* (MBTOC, 2014). Time needed for mortality at extreme temperatures has been shortened previously by the combination with the use of ice-nucleating bacteria to accelerate freezing at low temperatures (Lee *et al.*, 1992, Fields, 1993), fumigation (Yokoyama *et al.*, 1999, Fields & White, 2002, MBTOC, 2014), and controlled atmospheres (i.e., low oxygen) (Fields & White, 2002, Mitcham *et al.*, 2006, Denlinger & Lee 1998). Future studies should determine the effects of cold/heat combined with other methods of control on the survival of *T. granarium* and on the quality of the treated commodities.

Further understanding of the biology of *T. granarium* including de-acclimation, diapause, and its microbiome might allow their manipulation to design more effective methods of

control (Fields, 1992, Fields *et al.*, 1998). For example, the bacterium *Pseudomonas syringae* expresses an ice-nucleating protein (INA) in its membrane, which promotes ice crystals formation (freezing) of supercooled water (Cochet & Widehem, 2000). This principle was used to express the INA gene in *Enterobacter cloacae*, a bacterium in the microbiome of the mulberry pyralid larvae, *Glyphodes duplicales* (Lepidoptera: Pyralidae) (Watanabe *et al.*, 2000). The supercooling point (SCP) increased from −11.3°C (control fed with distilled water) and −4.7°C (larvae fed on *P. syringae*) to −3.3°C when the insect was fed on the transgenic bacteria (Watanabe *et al.*, 2000). This effect was due to the ability of *E. cloacae* to thrive in the insect's digestive tract (Watanabe *et al.*, 2000). The further understanding of *T. granarium* might lead to similar techniques to the ones used for the mulberry larvae to decrease the time necessary to reach mortality at extreme temperatures (Beard *et al.*, 2002, Panagiotis & Bourtzis, 2007). The removal of beneficial members of the microbiome and the manipulation of the reproductive effects of *Spiroplasma* on *T. granarium* may potentially reduce the population of this insect as it has been successfully done in mosquitos infected with *Wolbachia* (Panagiotis & Bourtzis, 2007, Bourtzis *et al.*, 2014). The results of this thesis provide an insight into the thermal tolerance and the microbiome composition of one population of *T. granarium*. Further research is needed considering more populations from different geographic locations and genetic background to confirm whether these results can be applied to populations of other countries.

Conclusions

The successful design and application of methods of control against *T. granarium* requires the understanding of its biology. The use of extreme temperatures to control *T.*

granarium should consider the effects of acclimation and diapause, which according to the results of this thesis greatly increase thermal tolerance. This thesis also identified high quantities of *Spiroplasma* bacteria in *T. granarium*, which might affect the insect's biology and tolerance to extreme conditions. The identification of *Spiroplasma* in *T. granarium* is a starting point and opens a new field of questioning attempting to understand this specific interaction and its applications. Further research is needed to understand and manipulate *T. granarium* biology, in order to develop, integrate, and apply different control tactics for this pest.

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