INDUCTION AND TERMINATION OF DIAPAUSE IN KHAPRA BEETLE, *TROGODERMA GRANARIUM*

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DEDICATION

To my parents, Shivananjappa and Manjula, for being incredibly supportive.

ABSTRACT

Larvae of the stored product pest, khapra beetle, *Trogoderma granarium*, are unusual in that they can enter a diapause state that confers protection against pest control methods. In Chapter 1, I reviewed the literature on general aspects of insect diapause induction and termination, particularly in Dermestidae. In Chapter 2, I assessed the effects of age of culture and diet quality on diapause termination. The results indicated that diapausing larvae can accumulate the nutrients required to terminate diapause and complete development when provided with an opportunity to do so. In Chapter 3, I assessed whether desiccation enhances cold tolerance in diapausing and non-diapausing larvae. Results showed that cold tolerance of diapausing larvae increased with greater desiccation stress. This finding suggests that the physiological mechanisms that protect diapausing larvae from desiccation may also increase cold-tolerance. Overall results of this research provide new information with potential application for control of this global pest.

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LIST OF ABBREVIATIONS

Symbol	**Definition**
%	Percent
±	Plus or minus
°C	Degree Celsius
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
cm	Centimetres
ddf	Denominator degrees of freedom
dia	Diameter
DL	Diapausing larvae
FD	Fresh diet
g	Grams
GLM	Generalized linear model
ht	Height
Lt	Lethal time
mL	Millilitre
mm	Millimetre
ndf	Numerator degrees of freedom
NDL	Non-diapausing larvae
RH	Relative humidity
SD	Spent diet
SEM	Standard error of mean
TOR	Target of rapamycin
vs	Versus

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Objectives

Larvae of the stored product pest, khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), are unusual in that they can enter a diapause state. Diapause is a period of suppressed growth and development in insects. It is thought to have originally evolved because it can help individuals survive adverse environmental conditions. In pests such as *T. granarium*, it may also confer protection against pest control methods.

Diapause is often a temporary state, whose induction and termination is influenced by various intrinsic and environmental factors. To gain insights into how larval diapause can be induced or terminated to aid pest control efforts, this thesis undertakes three main objectives:

i. <u>To test the effects of crowding on diapause induction</u>: Larvae are reared at different densities to determine the effects of crowding on diapause induction. Results provide insights into how diapause can be induced and terminated to aid control of this pest.
ii. <u>To evaluate the effect of diet qualities and duration in diapause on diapause termination</u>: Larvae that have been in diapause for different periods of time will be fed diets of different nutritional quality. Results provide insight into the combination of diet quality and diapause depth on diapause termination.
iii. <u>To assess the effect of diapause and desiccation on the larval cold-tolerance</u>: Diapausing and non-diapausing larvae reared at different relative humidities will be assessed for cold tolerance at -10 °C. Results will clarify the complex relationship between diapause, cold-hardiness and desiccation.

1.2 Introduction

Arthropods that are pests of stored products include over 600 species of beetles, 70 species of moths and 335 species of mites (Rajendran, 2002). By feeding on and contaminating grains and other stored products, the economic losses associated with these pests are estimated to be several billion dollars annually (Channaiah *et al.*, 2010). Feeding damage can also foster the growth of micro-organisms (Sinha & Wallace, 1966) that may pose health risks such as pathogenic microbial infection (Hill, 1990) and allergic reactions (Campbell *et al.*, 2004).

Many species of dermestids (Coleoptera: Dermestidae) are synanthropic and have become economically important pests of stored products, aided by their ability to thrive under diverse ecological conditions (Rajendran & Hajira Parveen, 2005). Affected products include stored grains and cereal products, dried insects and mammal specimens in museums, dried blood, dead mice, stored silkworm cocoons, products containing wool, cotton, linen, synthetic fibres and nearly everything containing plant or animal materials (Hinton, 1945; Peacock, 1993).

Larvae of economically important species of dermestids usually undergo an obligate or facultative diapause that confers higher tolerance to extreme conditions (Table 1.1, Wilches *et al.*, 2016). Such conditions may include an unpredictable food supply, extended periods of starvation, extreme temperatures and desiccation (Wilches *et al.*, 2016).

Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), is one of the world's most economically important invasive grain and cereal product pests (Lowe *et al.*, 2000; Eliopoulos, 2013). It is established in Asia, Africa, the Middle East, and a few European countries. It is regulated as a quarantine insect in: Argentina, Australia, Azerbaijan, Bahrain, Belarus, Brazil, Canada, Chile, China, Kazakhstan, New Zealand, Paraguay, Russia, Ukraine, United States and Uruguay (Athanassiou *et al.*, 2019). Quarantine countries have stringent regulations to prevent importation of khapra beetle-infested products from countries with established populations. Widespread international trade with the movement of goods across the globe has increased the risk of distributing khapra beetle populations, threatening global food security (Ahmedani *et al.*, 2007; Athanassiou *et al.*, 2019). Following its discovery in California in 1953, the United States government implemented a 13-yr eradication program at a cost of US$ 15 million (US$ 128 million in 2019 dollars) (Kerr, 1981; Athanassiou *et al.*, 2019).

Depending on conditions, egg-to-adult developmental time for khapra beetle may range from 26 days to more than a year, with 10 to <1 generation per annum (Hadaway, 1956; Lindgren & Vincent, 1959). The eggs are 0.7 mm by 0.25 mm long, oval in shape, dull white in colour, and, in the context of grain industry, are laid between the grains or in the cracks and crevices of storage structures (Lindgren & Vincent, 1959). Eggs hatch in about 9-12 days. Larvae are 1.6-4.5 mm long, 0.25-1.5 mm wide with long tufts of hairs on the posterior end (Lindgren & Vincent, 1959). The duration of a larval instar is 5-7 days with six instars for females and five for males (Burges, 1957). With sufficient food, at moderate densities and under optimal conditions (30-37 ºC and 40-60% RH), the larval stage lasts for 33 days (females) and 28 days (males) for non-diapausing individuals (Burges, 1957; Lindgren & Vincent, 1959). However, diapause can be triggered by high densities and insufficient food. In such cases, individuals may persist as larvae for more than four years and pass through 4-15 instars (Burges, 1957; Burges, 1962a; Burges, 1963; Athanassiou *et al.*, 2019). The pupa is a quiescent stage and may last for 2-5 days. The adults are oval in shape and measure between 1.6-3.0 mm in length and 0.9-1.7 mm in width. Adults live for about 2-5 days and do not fly or feed (Hadaway, 1956).

Larval diapause is an important reason why khapra beetle has become a global pest. Arthropod diapause serves one or more of three functions; survival of food shortage, survival under adverse environmental conditions (e.g., winter), and synchronization of adult emergence (Andrewartha, 1952). It serves all three in khapra beetle (Burges, 1962a). Diapausing larvae survive long periods without food, a situation that arises when warehouses and malt storage facilities are left empty during offseason. The survival of larvae allows populations to persist until food again becomes available in these facilities; when this occurs diapausing individuals can break diapause, breed rapidly, and quickly expand their population. This is the most important function of diapause in this species (Burges, 1959b; 1962a). Diapause, in combination with the cryptic nature of larvae and adults, allows them to survive undetected for long periods in shipping containers and vessels carrying cargo to other countries (Hurlock, 1961; Bell, 1994; Athanassiou *et al.*, 2019).

Non-diapausing larvae continually feed and accumulate large quantities of metabolites, mainly in the form of lipids (Karnavar & Nair, 1969a). These stored materials are utilized for energy production during food scarcity and for egg or sperm production once the larvae become adults. Hence, adult fecundity is commensurate with the amount of reserve materials in their body (Karnavar, 1972; Karnavar, 1973; Karnavar, 1984). Adults that have passed through diapause are larger and have higher fecundity than their non-diapausing counterparts, which promotes a rapid population growth post-diapause (Karnavar, 1972; Gothi *et al.*, 1984; Karnavar, 1984). Diapause can also affect pheromone production by females and the associated male response (Bell, 1994). Post diapause, males have reduced attraction to female pheromones and the reduced response is proportional to the duration of larval diapause (Gothi *et al.*, 1984). This, in turn, may severely affect trap catches in warehouses and lead to erroneous pest monitoring results.

Diapausing larvae have enhanced survival to many of the common insecticides and fumigants effective against other major stored-product insect species, and also to extreme temperatures (Ghimire *et al.*, 2017; Kavallieratos *et al.*, 2017; Kavallieratos & Boukouvala, 2019). The most commonly used fumigant for control of khapra beetle is methyl bromide. However, this fumigant is being phased out and used only for quarantine treatments (Fields & White, 2002; Wilches *et al.*, 2017). Even for quarantine treatments, the recommended rates of methyl bromide applications are twice as high as compared to other stored-product pests (Athanassiou *et al.*, 2019). Diapausing larvae are also cold-and-heat-tolerant. Diapause and cold acclimation enhance its cold-hardiness, which requires exposure of 347 days at −10 °C to kill all individuals (Wilches *et al.*, 2017).

The type of dormancy exhibited by dermestids, particularly in khapra beetle, is unique and differs widely from the classical definition of diapause (Burges, 1959a; Wilches *et al.*, 2016). It has characteristics of both quiescence and diapause under varying environments. The dormancy appears to be quiescence when diapause is terminated by provisioning fresh food or when optimal temperatures for breeding are restored (Burges, 1963; Nair & Desai, 1973). However, not every individual within a population resumes development or breaks diapause when the above criterion is satisfied. Further, dormancy may last many months and even years if temperature is maintained at 30 °C, which cannot be regarded as adverse or unfavourable for growth and development (Burges, 1959a). Thus, this dormancy appears to be more complicated than quiescence (Burges, 1959a; Tauber *et al.*, 1986; Koštál, 2006; Wilches *et al.*, 2016). However, most researchers have treated the dormancy of khapra beetle to be facultative diapause.

My MSc research is mainly focussed on induction and termination of diapause in khapra beetle. Although there have been several studies on induction of diapause (Burges, 1963; Nair & Desai, 1972), little is known on the factors that terminate it. In the current chapter, I review general information about insect diapause induction and termination, particularly in Dermestidae. Because of their association with diapause, I also review the concept of cross-tolerance between cold and desiccation in insects and different physiological responses associated with it. I conclude with a brief outline of chapters included in this thesis.

1.3 The phenomenon of diapause

Dormancy is a physiological suspension or suppression of an organism's metabolic activity (Gordh & Headrick, 2011). In insects, dormancy can occur in egg, immature larval, pupal, and adult stages (Danks, 1987).

1.3.1 Diapause vs quiescence

In his review of dormancy, Danks (1987) distinguishes between diapause and quiescence. "Diapause is an adaptive state of arthropod physiological dormancy, often referred to as hibernation (winter diapause) or aestivation (summer diapause) in response to seasonal adverse environmental conditions" (Danks, 1987). It can also be defined as "a genetically programmed physiological state of arrested metabolic activity that enables an insect to survive under predictable adverse climatic conditions and diminishing resources needed for development, reproduction and morphogenesis" (Tauber *et al.*, 1986). In contrast, "quiescence is a type of irregular dormancy (non-seasonal) characterized by slowed metabolism and directly resulting from unfavourable environmental conditions" (Danks, 1987). However, quiescence is a less complex attribute as its initiation does not rely on endogenous control. A short-term inactive phase distinguishes quiescence as an immediate reaction to hostile environmental conditions, with development resuming shortly after favourable conditions arrive. Conversely, diapause is an endogenously regulated arrest that takes the organism away from normal metamorphosis to an alternative developmental pathway that does not necessarily resume when benign environmental conditions return (Andrewartha, 1952; Saunders, 1982; Danks, 1987).

1.3.2 Phases of diapause

Wheeler (1893) originally coined the term 'diapause' to describe a phase in the embryo morphogenesis of the grasshopper *Xiphidium* sp., yet it was first utilized in referring to dormant stages and developmental arrests in insect life cycles by Henneguy (1904). It since has been used in various contexts by different researchers. As of late, the inclusiveness of the phenomenon covered by the term diapause has been expanding. Because of the dynamic nature of diapause, Koštál (2006) reviewed the most commonly used terminologies and re-characterized the best-corroborated phases that incorporate the broad aspects of diapause.

The term "diapause development" was first instituted by Andrewartha (1952) to accentuate diapause as a "process" as opposed to as a "status". However, it was confusing to use the word "development" to depict a "developmental arrest" (Hodek, 2002). Thus, Koštál (2006) characterized diapause as a three-stage process with more logical terms for diapause initiation, maintenance, and termination.

Direct development (morphogenesis) stops during the diapause initiation phase, trailed by controlled metabolic suppression. Life stages that are mobile (larvae and adults) and capable of diapause continue to feed, form energy reserves, and look for suitable microhabitat. Diapause intensity increases to prepare for impending adversity (Koštál, 2006).

Amid the maintenance stage, endogenous developmental arrests occur while natural conditions for direct development are suitable. Precise token-stimuli help maintain diapause and the rate of metabolism drops or stays consistent. Undetermined physiological processes decrease diapause intensity and increase sensitivity to diapause terminating conditions (Koštál, 2006).

During the termination phase, when specific environmental conditions change, the diapause intensity is reduced to its minimum. A physiological state is attained, where a possibility for direct development is re-established (Koštál, 2006).

Although the physiological differences between the three phases of diapause are clear, there is disagreement about when transitions occur, particularly with respect to termination of diapause (Koštál, 2006). First, in general, there is no complete suspension of development during diapause (Hahn & Denlinger, 2007). Second, conflicting responses may occur due to exogenous ecological elements (e.g., temperature, photoperiod, relative humidity) during diapause (Gray *et al.*, 1995; Koštál, 2006). Third, there is always bio-chemical activity, even at lower metabolic rates (Danks, 1987). Fourth, despite the arrest of morphogenesis, during diapause, mobility, feeding, and growth may still take place (Hodek, 2002). Fifth, the diapause-based physiological mechanisms between species are highly variable. This is because diapause has evolved several times independently and can occur at various stages of development (Tauber *et al.*, 1986; Danks, 1987). These reasons make the generalization of these principles complicated for universal application across taxa.

1.3.3 Physiological changes associated with diapause

Insects entering diapause experience various physiological changes that increase metabolic reserves and decrease the use of energy and oxygen (Denlinger, 1991). This is due to diapause-specific gene activation that affects heat shock, energy metabolism and storage, hormonal regulation and diapause-induced clock proteins (Koštál, 2006; Wilches *et al.*, 2016).

Diapausing and non-diapausing insects store metabolic reserves (e.g., lipids, carbohydrates, and amino acids) and essential micro-nutrients (e.g., vitamins and minerals) (Hahn & Denlinger, 2011). Cellular maintenance reserves are used during diapause for catabolic and anabolic energy generation, protein turnover, cell membrane maintenance and reconstruction (Hahn & Denlinger, 2011). The dominant forms of energy storage are triacyl-glycerides, but some hexameric proteins and reserves of glycogen are often accumulated (Hahn & Denlinger, 2011).

Internal pre-diapause reserves collected are critical to the supply of metabolic substrates during and after diapause (Danks, 1987; Hahn & Denlinger, 2007). Such reserves are essential in maintaining the metabolic activity of non-feeding life stage (eggs, pupae, or wandering larvae) reserves (Tauber & Tauber, 1976; Tauber *et al.*, 1986), and also for feeding life stages whose nutrient intake during diapause can be absent or reduced.

Signalling pathways and hormones also regulate diapause. Insulin signalling regulates the storage of diapause energy reserves and may be important in integrating metabolism regulation during pre-diapause and diapause. If so, this pathway would likely influence life-history traits and post-diapause processes. The target of rapamycin (TOR) signalling, AMP-activated protein kinase (AMPK), and insect adipokinetic hormones are other pathways and hormones that influence diapause nutrient reserves (Harvey, 1962; Denlinger, 2002; Hahn & Denlinger, 2007; Hahn & Denlinger, 2011).

1.4 Diapause in Dermestidae

The majority of the stored-product insects are species of Coleoptera or Lepidoptera. Diapause (obligate or facultative) in Coleoptera has been observed for species of Bruchidae, Dermestidae, Ptinidae and Tenebrionidae (Bell, 1994). Diapause in Lepidoptera has been reported mainly for species of Pyralidae and for a few species of Gelechiidae, Oecophoridae and Tineidae (Bell, 1994). The first report of diapause in a stored-product insect was for *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae) (Richards & Waloff, 1946), with subsequent extensive studies on *T. granarium* (Burges, 1957; 1959a; Burges, 1959b; Burges, 1959c; 1960; 1962b; 1963).

Dermestidae currently comprises about 1200 known extant species (45 genera in 12 tribes) that occur in every zoogeographical region of the world (Háva, 2004; Wilches *et al.*, 2016). Dermestid larvae feed on the desiccated tissues of dead vertebrates and invertebrates. In nature, they are associated with spider webs, lepidopteran cocoons, and in the nests and dens of Hymenoptera, mammals and birds (Háva, 2004; Zhantiev, 2009). Dermestid species feed either on desiccated corpse tissues or on nectar and pollen, but adults of a few species prey on the larvae of bees and wasps, and on spider eggs (Háva, 2004; Wilches *et al.*, 2016).

Species of *Anthrenus* and *Attagenus* have a generation time of 1-3 years with an obligate larval diapause (Bell, 1994). *Anthrenus verbasci* Linnaeus has two bouts of dormancy, as early instar and late instar larvae (Blake, 1958; 1959). Development can take 1, 2 or 3 years in the fully grown larvae with obligate diapause (Blake, 1958; 1959; 1961). Slower-developing individuals and under-developed larvae defer development from 10-15 weeks age at lower temperatures, which matches with the onset of winter, a character typical of true diapause (Bell, 1994). However, in the closely-related species *Anthrenus sarnicus* Mroczkowski and *Anthrenus* *flavipes* LeConte, only mature larvae diapause (Griswold & Greenwald, 1941; Armes, 1988; 1990). An "internal clock" regulates diapause in these three species; i.e., all individuals pass through diapause, but photoperiod and temperature affect its timing and duration (Blake, 1959; Armes, 1988; 1990; 1991).

Species of *Trogoderma* can undergo an arrested state of development with characteristics similar to the diapause of *T. granarium* with few dissimilarities. The proportion of *T. variabile* larvae entering diapause increased when temperature was decreased from 32 °C (4-11 °C) (Loschiavo, 1960). *Trogoderma variabile* larvae enclosed in a limited living space at low densities did not pupate even with an ample amount of fresh food (Burges, 1961). Multiple strains of *T. variabile* entered diapause when reared in isolation at constant temperatures (27 °C or 32 °C) (Partida & Strong, 1975). Food volume, photoperiod, temperature and density of larvae (isolation) acted alone or in combination to induce diapause in *T. variabile* (Wright & Cartledge, 1994). About 92% of *T. variabile* larvae entered diapause when reared under isolation at 30 °C (Abdelghany *et al.*, 2015). Other species of *Trogoderma* that undergo facultative diapause under isolated conditions include *Trogoderma anthrenoides* Sharp (Burges & Cammell, 1964), *Trogoderma glabrum* Herbst (Archer & Strong, 1975), *Trogoderma simplex* Jayne (Strong & Mead, 1975b), *Trogoderma inclusum* LeConte (Strong, 1975b), *Trogoderma sternale* Jayne (Bell, 1994), and *Trogoderma grassmani* Everts (Bell, 1994).

Beck (1971a) studied the phenomenon of "retrogressive moulting" in larvae of the glabrous carpet beetle *T. glabrum* (Beck, 1971a; 1971b; 1972; 1973a; 1973b). Under optimal conditions (30 °C, 65-70% R.H.), larvae underwent five (males) or six (females) instars. Male growth period ranged from 20 to 30 days and female growth period from 32 to 36 days, with females undergoing supernumerary 7th larval stadium to delay pupation (Beck, 1971a). When deprived of food, female larvae of the 6th instars did not pupate and entered a period of retrogressive larval growth, sporadically shedding larval skins to become progressively smaller size and mass. Larvae retro-molted five to eight times during one year of total food deprivation (Beck, 1971a; 1971b; 1972; 1973a; 1973b).

Beck (1971a) differentiated between three physiological states; normal, dauer, and retrogressive larval development. Dauer larvae underwent supernumerary moults, but did not metamorphose due to poor quality food, disturbance or isolation. As a result of food deprivation, the size of the retrogressive larvae decreased with subsequent moults, further characterized by reduced oxygen uptake. The pupation of these larvae was inhibited by small rearing containers and absence of male pupae, and was stimulated by high population density. Female larvae, which had several retrogressive moults, regained weight and matured rapidly when food was supplied. In addition, the regrown larvae did not pupate without exposure to long photoperiods or male pupae of the same species (Beck, 1971a; 1971b; 1972; 1973a; 1973b). For this remarkable capacity of retrogressive moulting, *T. glabrum* is also referred to as “ The Benjamin Button of The Insect World” by entomologists (Ballenger, 2014).

The biological significance of retrogressive development is puzzling. Retrogressive moulting occurs during periods of starvation, yet expends energy and results in the loss of valuable metabolites and external cuticular layers. This phenomenon would, therefore, appear to further jeopardize the insect’s survival. Thus, the significant adaptive advantage of “retrogressive development” needs further investigation (Beck, 1971a; Beck & Bharadwaj, 1972). This is particularly so for khapra beetle because of its economic importance.

1.5 Diapause in khapra beetle

Researchers have interpreted the phenomenon of diapause in khapra beetle in different ways. Barnes & Groove (1916) first referred to this as a “hibernating condition”; e.g., when most matured larvae did not pupate at low temperatures for several months. Hadaway (1956) referred to this phenomenon as a “state of quiescence” from the growth caused by lower temperature and other factors. Lindgren & Vincent (1959) referred to it as “short-lived and long-lived”, but extensive studies by Burges (1957; 1959a; 1959b; 1959c; 1960; 1962b; 1963) clarified this phenomenon and made meaningful interpretations, conveniently referring to this as “facultative diapause”.

1.5.1 Factors responsible for initiation of diapause

Several factors induce diapause in mature fifth-instar khapra beetle larvae. Such factors may act alone or in combination and include: i) larval density, ii) temperature, iii) relative humidity, iv) presence of fecal pellets, and v) diet quality (Burges, 1963; Nair & Desai, 1972).

1.5.1.1 Role of larval density

Larval crowding is known to induce diapause or inhibit pupation in several species of insects (Maclagan, 1932; Edward, 1958; Tschinkel & Willson, 1971; Barak & Burkholder, 1977b; Nakakita, 1982; Kotaki *et al.*, 1993; Togashi, 2017). Mature khapra beetle larvae delay pupation and enter diapause under high larval densities at 30 °C (Burges, 1959b; Burges, 1959c; 1960; 1962b). Diapause is instigated by joint action of temperature and larval density at or below 30 °C. In his research, Burges (1963) found that all larvae (100%) enter diapause at 25 °C in crowded cultures (25 larvae/g diet) and about 50% at 30 °C and about 2-3% at 35 °C and 40 °C. There is no diapause under isolation and the proportion of diapausing larvae increase with increased larval densities. Hence, the diapause of khapra beetle is regarded as a density-dependent phenomenon. Somewhat in contrast to the findings by Burges (1963), Nair & Desai (1972); Wilches *et al.* (2017) report that 32% of isolated larvae and 90% of crowded cultures of 10 larvae per 300 mg diet (equivalent to 33 larvae per 1 g diet) exhibit diapause when reared at 30 °C.

1.5.1.2 Role of temperature

Temperature plays a pivotal role in initiation of diapause in khapra beetle. Any temperature below the normal breeding range of 21-41 °C induces diapause regardless of other factors (e.g., larval density, relative humidity). However, diapause induction is greatest at temperatures at or below 30 °C in combination with higher larval densities (>33 larvae/g of diet) (Burges, 1963; Nair & Desai, 1972; Burges, 2008).

1.5.1.3 Role of humidity

Humidity is a potential factor in diapause induction, but its mode of action has not yet been identified in any insect capable of diapause (Masaki, 1980). Initially, researchers did not test for the effect of humidity on diapause induction. Shulov (1955) ascertained normal khapra beetle development and reproduction when reared at 80-90% relative humidity (RH). Lindgren *et al.* (1955) noted increased mortality of larvae and pupae at higher humidities. Humidities below 25% were detrimental to larval development (Hadaway, 1956). Burges (1959b) studied the ecology of khapra beetle in malt stores and found that a sharp gradient of moisture between the outer and inner layer of the stored-product did not affect larval behaviour. To investigate the effect of relative humidity on diapause induction in a separate experiment, Burges (1963) reared larvae at four different relative humidities; 0, 35, 70, and 90% and concluded that relative humidities of 0% and 90% increased the proportion of diapausing larvae. Spangler (1965) found that larvae avoided high-humidity environments. Yinon & Shulov (1969) studied the humidity gradient response and concluded that both males and females exhibit a hygro-negative response.

1.5.1.4 Role of fecal pellets

While examining the mechanism of larval density, Burges (1963) noted that fecal pellets in the spent media when blended with a mixture of whole and damaged grains induced diapause even when larvae were isolated. He attributed this to an unknown mechanism associated with the transfer of a chemical from the fecal pellets to the food. However, subsequent research revealed the effect of fecal pellets inducing diapause when diluted with inert materials (i.e., cellulose powder, bacto-agar, sawdust and sand) (Stanic *et al.*, 1963; Stanic & Shulov, 1969). When mixed with food, ether extractable fecal pellet lipids induced diapause in larvae reared in isolation, but not to the extent observed when intact fecal pellets were mixed with food (Karnavar & Nair, 1969b). Subsequently, Ikan *et al.* (1970) concluded that dietary fatty acid fraction of the fecal material contained palmitic, stearic, oleic and linoleic acids that induced and maintained diapause. Ikan *et al.* (1970) further confirmed that linoleic acid, either by external feeding or by infusion was highly efficient in inducing diapause.

1.5.1.5 Role of diet quality

Diet quality is also known to influence diapause induction. Nair & Desai (1972) observed diapause induction by rearing larvae in isolation on sub-optimal diets. When larvae were fed with wetted and dried flour of maize or wheat, the majority showed extreme developmental impediments and immediately entered diapause. Higher temperature (<35 °C) and yeast supplementation nullified this effect, which demonstrated that suboptimal diets induce greater diapause at diapause-promoting temperatures. Wheat flour treated with methanol and chloroform had a comparable impact, but methanol treatment alone had no effect. Accordingly, Nair &

Desai (1972) classified diapause into three categories; (1) DID (Density-Independent Diapause) – diapause that is induced in isolation; (2) DDD (Density-Dependent Diapause) – diapause induced in crowded cultures due to increasing larval density; (3) NDD (Nutrition-Dependent Diapause) – diapause induced by fecal components, such as lipids extracted from ether or linoleic acid.

1.5.2 Factors responsible for terminating diapause

Although several researchers have described the factors that induce diapause, only cursory information is available on the factors that terminate it. According to Burges (1963), diapause can be terminated to varying degrees by three treatments, regardless of the method of diapause induction: (1) a considerable increment in temperature within the breeding range (21-41 °C); for instance, return of diapausing larvae from 20 to 30 °C or from 30 to 35 °C (highly efficient), (2) temperature treatments below 21 °C for a month followed by return to warm conditions (very efficient) or (3) providing fresh diet (less efficient). Likewise, Nair & Desai (1973) found that the larvae broke diapause under following conditions: (1) on encountering a significant increase in temperature, after entry into a specific time-frame in the absence of environmental stimuli, or (2) when supplied with new diet in combination with decreased larval crowding. Nair & Desai (1973) further stated that density-independent diapause could not be broken even when fresh food was provisioned under optimal growth conditions, speculating that some sort of endogenous rhythm influenced diapause termination.

1.6 Cold and desiccation tolerance

In insects, almost all activities are influenced by temperature, determining the status of species activity and geographic distribution. In a broader sense, these include cellular enzymatic reactions, various physiological processes, behaviour, and evolution (Lee, 1991). For stored-product insects, temperatures of 25-33 ºC are optimal for normal development and reproduction, and most insects from this group can complete their development at temperatures as low as 13 ºC or as high as 35 ºC (Fields, 1992). Temperatures below 13 ºC and above 35 ºC are not favourable for development of these insects. Longer durations and more extreme temperatures cause faster and greater mortality (Fields, 1992).

All of the stored-product insects investigated so far are freeze-intolerant and avoid death at sub-zero temperatures by avoiding freezing (Wilches *et al.*, 2017). Based on survival at freezing temperatures, two fundamental strategies of cold-hardiness have been well-described in literature; freeze-intolerant and freeze-tolerant insects (Storey & Storey, 1988; Fields, 1992). Freeze-intolerant insects die with the formation of ice within their bodies, whereas freeze-tolerant insects can withstand extra-cellular freezing (Storey and Storey 1988; Fields 1992).

Desiccation is a significant environmental stress that confronts overwintering insects. Among desiccation and numerous other environmental stressors encountered by insects, cold and desiccation stress are particularly believed to be governed by overlapping sets of regulatory and mechanistic processes (Sinclair *et al.*, 2013). Several studies report that cold and desiccation tolerance share many common traits such that the two stressors have a degree of cross-tolerance (Ramlov & Lee, 2000; Bayley *et al.*, 2001; Sinclair *et al.*, 2013; Terhzaz *et al.*, 2015). Solutes in the hemolymph, habitat selection and protective ecological structures play a dual role by conferring protection to both desiccation and cold (Ring & Danks, 1998). These protective mechanisms have been jointly referred to as “cross-tolerance” by Sinclair *et al.* (2013). Other adaptations to cold and desiccation stress include signalling pathways (“cross-talk”) and responses at cellular, molecular and biochemical levels (Sinclair *et al.*, 2013). However, possible mechanisms for such inter-linked responses remain poorly understood (Sinclair *et al.*, 2013; Terhzaz *et al.*, 2015).

A common response to cellular dehydration - a primary mode of damage due to desiccation or freezing - is the production of low molecular weight compounds called cryoprotectants (Ring, 1994; Ring & Danks, 1998). These cryoprotectants are generated as a result of gene expression whose functions overlaps to confer protection against desiccation and cold-related injury. A few of these genes are responsible for the production of antifreeze proteins and trehalose that help preserve protein and membrane structure, thereby improving tolerance to desiccation or cold (Sinclair *et al.*, 2013). However, in some cases, increased cold and desiccation tolerance might be associated with diapause (Denlinger, 1991; Danks, 2000), but the relationship is unclear and remains controversial (Denlinger, 1991; Pullin, 1996). Moreover, adaptations that allow these insects to resist cold and desiccation may be coincidental with adverse environmental conditions, and may not be controlled by diapause alone (Danks, 2000). Therefore, species of tropical origin, with diapause in their life cycle, may be important model organisms for deciphering the interaction between diapause, cold and desiccation.

The khapra beetle and the granary weevil *Sitophilus granarius* Linnaeus (Coleoptera: Curculionidae) are among the most cold-tolerant stored-product insects investigated for cold tolerance (Fields, 1992; Wilches *et al.*, 2017). This finding for khapra beetle is fascinating, given that it is a species of tropical region (hot and arid regions) from the Indian sub-continent. Other insect species that are extremely cold hardy are mostly from temperate polar regions (Turnock & Fields, 2005; Hayward *et al.*, 2007). Hence, there is speculation that khapra beetle's remarkable cold-hardiness may be due to cross-tolerance with desiccation (Sinclair *et al.*, 2013); i.e., because of its adaptation to dry and hot climates, it could be pre-adapted to cold environments (Wilches *et al.*, 2017). Thus, additional experiments are necessary to test the notion of cross-tolerance and clarify the complex relationship between diapause, cold-hardiness and desiccation.

Bacterial symbionts also may confer cold and desiccation tolerance to stored product insects. The symbionts *Sulcia* and *Blattabacterium* affect cuticular thickness, melanization and hydrocarbon profile in saw-toothed grain beetle *Oryzaephilus surinamensis* Linnaeus (Coleoptera: Silvanidae) (Engl *et al.*, 2018). These symbionts enhanced desiccation resistance and improved fitness under dry conditions (Engl *et al.*, 2018). The authors speculate that bacteriome-located intracellular symbionts confer desiccation resistance through ancient symbiosis. This finding correlates with another novel discovery by Wilches *et al.* (2018), who reported that the microbiome of khapra beetle was dominated by *Spiroplasma* bacteria. However, the role of *Spiroplasma* in this species is unknown. Thus, further research is necessary to identify the role of *Spiroplasma* in khapra beetle to manage this invasive pest successfully (Wilches *et al.*, 2018).

1.7 Summary

The majority of the key literature on khapra beetle was produced more than 40 years ago, and there are serious gaps in key biology issues and modern control of this species, especially with regards to diapause termination (Athanassiou *et al.*, 2019). Despite the fact that there are several publications on khapra beetle during the last years, most authors have not summarised the key aspect of its biology; i.e., larval diapause. Thus, this chapter is an attempt to summarise and present a comprehensive review of diapause in stored-product Dermestidae with a special emphasis on khapra beetle. This review covers various aspects of diapause in insects such as differences between diapause and quiescence, diapause phases and theories, and physiological changes associated with diapause. It also covers diapause in stored-product Dermestidae, examining the factors responsible for diapause induction and termination. Finally, it presents various relationships and physiological changes associated with insect tolerance for cold and desiccation.

1.8 Structure of thesis and goals of each chapter

Chapter 2 Effect of diet quality and age of culture on diapause termination in khapra beetle, *T. granarium*

Chapter 2 describes two major experiments (1) the effect of diet quality and age of culture on diapause termination, mortality and larvae in diapause at six months, and (2) the effect of khapra beetle infestation on diet parameters such a moisture, pH, dry matter, crude protein, crude fat, ash, and fibre. The first experiment is designed to understand the influence of diapause depth on diapause termination and to examine the effect of larval feeding on various bio-chemical parameters of khapra diet.

Chapter 3 Cross-tolerance to desiccation and cold in khapra beetle, *T. granarium*

Chapter 3 describes two experiments to assess the larval cold-tolerance of diapausing and non-diapausing khapra beetle obtained on four different relative humidities created using saturated salt solutions. In the first experiment, the effect of density of larvae on the proportion of diapause was assessed at 30 °C to obtain larvae in diapause for investigating cold-tolerance. Non-diapausing larvae were obtained by low-density rearing (25 larvae/6 g diet) for 18 days after hatch, and diapausing larvae were obtained by densities greater than 220 larvae/6 g diet for 45 days after hatch. In the second experiment, cold-tolerance was assessed by exposing non-diapausing and diapausing larvae to -10 °C for different durations to calculate lethal times for

50% and 95% of the population (LT_{50} and LT_{95}) and examine the relationship between cold and desiccation tolerance in khapra beetle.

Chapter 4 Discussion and Conclusions

Chapter 4 synthesizes the overall findings and general conclusions for Chapters 2 and 3. The contribution of the thesis results for the biology and management of khapra beetle, recommendations and future approaches are discussed.

Table 1.1 Economically important dermestids that undergo diapause in their life cycle*

Common name	Scientific name	Diapause	Reference
Asian carpet beetle	*Anthrenus coloratus* (Reitter, 1881)	Yes	(Ali, 1997)
Furniture carpet beetle	*Anthrenus flavipes* (LeConte, 1854)	Yes (obligate)	(Griswold & Greenwald, 1941; Ayappa *et al.*, 1957)
Guernsey carpet beetle	*Anthrenus sarnicus* (Mroczkowski, 1963)	Yes (obligate)	(Coombs & Woodroffe, 1983; Armes, 1988)
Varied carpet beetle	*Anthrenus verbasci* (Linnaeus, 1767)	Yes (obligate)	(Griswold & Greenwald, 1941; Blake, 1958)
Australian carpet beetle	*Anthrenocerus australis* *(Hope, 1843)*	Yes (obligate)	(Gerard & Ruf, 1997)
-	*Attagenus augustatus* *(Ballion, 1871)*	-	(Veer & Rao, 1995)
-	*Attagenus cyphonoides* (Reitter, 1880)	Yes (obligate), larvae overwinter	(Veer *et al.*, 1991)
"Yellow" black carpet beetle	*Attagenus elongatulus* (Casey, 1900)	Yes (crowded)	(Barak & Burkholder, 1977a)
-	*Attagenus indicus* (Kalík, 1954)	Hibernates as larvae or adult	(Veer & Rao, 1995)
Black carpet beetle	*Attagenus unicolor* (Brahm, 1791)	Yes (obligate and larval crowding)	(Back & Cotton, 1938; Griswold & Greenwald, 1941)
Wardrobe beetle	*Attagenus fasciatus* (Thunberg, 1795)	-	(Ali *et al.*, 2011)
-	*Attagenus lobatus* (*Rosenhauer, 1856)*	Overwinters as larvae or pupae	(Veer *et al.*, 1991)
Black carpet beetle	*Attagenus scalaris* (Pic, 1893)	-	(Abdel-Rahman *et al.*, 1981)
Brown carpet beetle	*Attagenus smirnovi* (Zhantiev, 1973)	Yes	(Stengaard Hansen *et al.*, 2012)
Black larder beetle	*Dermestes ater*	-	(Coombs, 1981)

	(DeGeer, 1774)		
Sheep skin dermestid	*Dermestes haemorrhoidalis* (Küster, 1852)	-	(Coombs, 1979)
Larder beetle	*Dermestes lardarius* (Linnaeus, 1758)	-	(Coombs, 1978; Jacob & Fleming, 1984)
Leather (Hide) beetle	*Dermestes maculatus* (DeGeer, 1774)	Yes (crowded)	(Osuji, 1975; Cloud & Collison, 1986)
Peruvian larder beetle	*Dermestes peruvianus* (LaPorte de Castelnau, 1840)	-	(Coombs, 1979)
-	*Trogoderma anthrenoides* Sharp, 1902	Yes (isolation)	(Burges & Cammell, 1964)
Warehouse beetle	*Trogoderma variabile* (Ballion, 1878)	Yes (isolation)	(Loschiavo, 1960; Partida & Strong, 1975)
Khapra beetle	*Trogoderma granarium* (Everts, 1898)	Yes (crowded, isolation)	(Hinton, 1945; Hadaway, 1956; Burges, 1962a)
European larger cabinet beetle	*Trogoderma versicolor* (Creutzer, 1799)	-	(Hadaway, 1956)
Larger cabinet beetle	*Trogoderma inclusum* (LeConte, 1854)	Yes (isolation)	(Strong, 1975a; Klein & Beck, 1980)
Cabinet beetle	*Trogoderma angustum* (Solier, 1849)	Yes (isolation)	(Bell, 1994)
Cabinet beetle	*Trogoderma sternale* (Jayne, 1882)	Yes (isolation)	(Bell, 1994)
Glabrous carpet beetle	*Trogoderma glabrum* (Herbst, 1783)	Yes (isolation)	(Beck, 1971a; Archer & Strong, 1975; Klein & Beck, 1980)
Ornate cabinet beetle	*Trogoderma ornatum* (Say, 1825)	Yes	(Bell, 1994)

-	*Trogoderma simplex* (Jayne, 1882)	Yes (isolation)	(Strong & Mead, 1975a)
Tiny cabinet beetle	*Trogoderma grassmani* (Beal, 1954)	Yes (isolation)	(Bell, 1994)

Blank cells indicate unavailable information

[*] Table modified from Wilches *et al.* (2016)

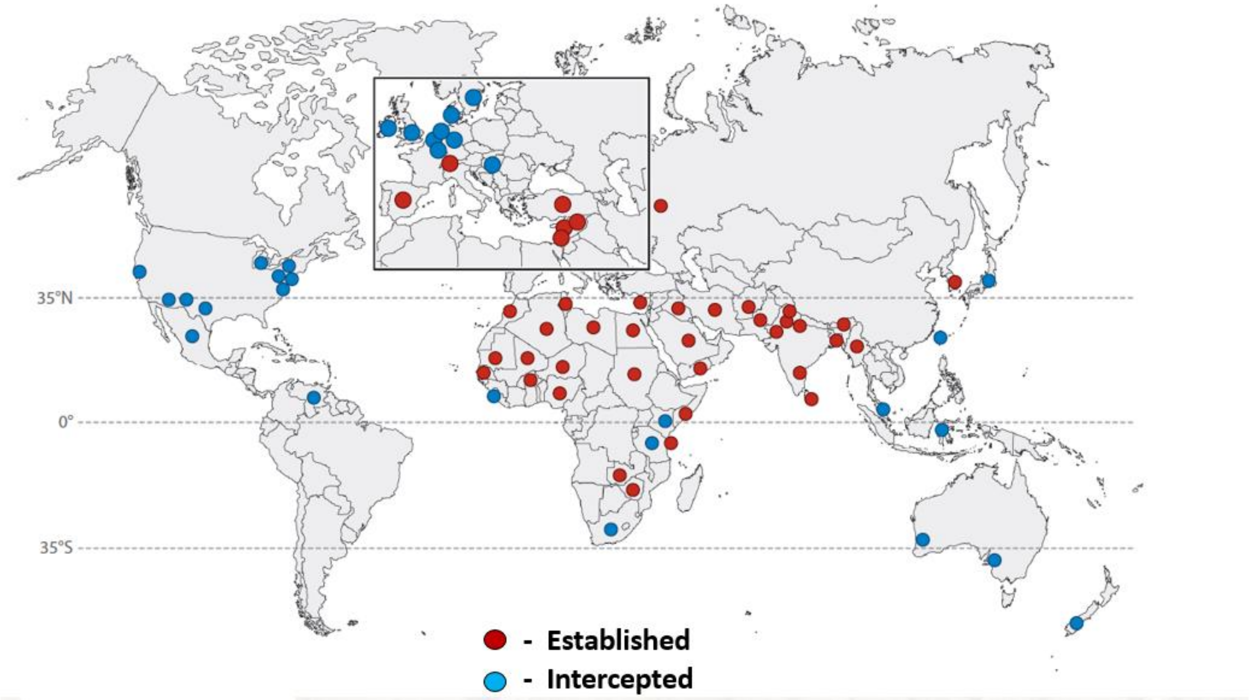

Figure 1.1 Map depicting the khapra beetle's current known world distribution by country, as set apart with red circles. Blue circles indicate countries that had infestations in the past century beyond ports of entry, but which are eradicated or for which there are no current records of occurrence. Figure adapted from Athanassiou *et al*. (2019).

CHAPTER 2: EFFECT OF DIET QUALITY AND AGE OF CULTURE ON DIAPAUSE TERMINATION IN KHAPRA BEETLE, *Trogoderma granarium*

2.1 Abstract

Larvae of khapra beetle, *Trogoderma granarium* (Coleoptera: Dermestidae) have an unusual diapause during which they may sporadically feed for several years, but not complete development. Diapausing larvae have an enhanced tolerance to starvation, insecticides and extreme temperatures. Thus, knowledge of factors that terminate larval diapause may aid in the control of this pest. In the current study, I assessed the effect of diet quality (five mixtures of fresh and spent diet in a replacement series) on diapause termination for diapausing larvae from laboratory cultures aged 3, 10, or 14 months. Larvae (*n* = 10 replicates for each combination of diet quality and culture, 10 larvae/replicate) were held at 30 °C and observed at different intervals over the course of 188 days to record larval mortality and larval pupation (= diapause termination). Larvae were least able to survive on diets of lower quality; i.e., 88% mortality after 188 days on 0% fresh diet versus 8% mortality on 100% fresh diet (averaged across cultures). Larvae were most likely to terminate diapause on diets of higher quality; i.e., 87% termination after 188 days on 100% fresh diet versus 0% termination on 0% fresh diet (averaged across cultures). Diapausing larvae from older cultures were least likely to terminate diapause and least likely to survive. On 100% fresh diet, diapause termination of larvae from cultures aged 3, 10 and 14 months was 93, 91, and 78%, respectively. On 0% fresh diet, mortality of larvae from cultures aged 3, 10 and 14 months was 67, 97, and 100%, respectively. These results indicate that diapausing larvae can accumulate the nutrients required to terminate diapause and complete development when provided with an opportunity to do so. However, the window of opportunity continues to shrink the longer the larvae remain in diapause.

2.2 Introduction

Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), is among the world's most economically-important pests of stored grains and cereal products (Lowe *et al.*, 2000; Eliopoulos, 2013). With populations established in Asia, Africa, the Middle East and parts of southern Europe, strict quarantine protocols are in place elsewhere to prevent its spread into countries that include Argentina, Australia, Azerbaijan, Bahrain, Belarus, Brazil, Canada, Chile, China, Kazakhstan, New Zealand, Paraguay, Russia, Ukraine, United States and Uruguay (Athanassiou *et al.*, 2019). Because failure to exclude the pest can reduce a country's export markets and local food availability (Ahmedani *et al.*, 2007; Athanassiou *et al.*, 2019), there is a strong incentive to eradicate khapra beetle populations when detected. Following its discovery in California in 1953, the United States government implemented a 13-yr eradication program at a cost of US$ 15 million (US$ 128 million in 2019 dollars) (Kerr, 1981; Athanassiou *et al.*, 2019).

Khapra beetle is a non-selective feeder that infests a wide array of common stored products. It mainly infests cereal grains and pulses, including several plant and animal materials, particularly seeds from many plant families (Hopkins, 1955; Rajendran & Hajira Parveen, 2005; Kavallieratos *et al.*, 2019). It is a serious pest of maize, sorghum, rice, barley, gram, pulses, pistachio nuts, and walnuts, and particularly wheat (Golizadeh & Abedi, 2016; Majd-Marani *et al.*, 2018; Kavallieratos *et al.*, 2019). It also infests non-grain dried commodities including herbs, spices, gums, orange pulp, coconuts (copra), fruits, and several other dried proteinaceous materials such as dried blood, dried insects and dead mice (Hinton, 1945; Rahman *et al.*, 1945; Girish *et al.*, 1975; Athanassiou *et al.*, 2016; Golizadeh & Abedi, 2016; Mohammadzadeh & Izadi, 2018; Athanassiou *et al.*, 2019; Kavallieratos *et al.*, 2019). In addition, the beetle has been found in many locations that are not sources of food, such as seams and ears of burlap bags and wrappers, baled crepe rubber, cars, steel wire, books, corrugated boxes (glue), bolt bags, and even soiled linen and oil paintings (Nair, 1972), which confers khapra beetle a status as an ‘omnivorous protein scavenger’.

Khapra beetle infestations in stored products significantly reduce their quality, weight and grade, making them unmarketable and unpalatable. Typically, larvae feed on the embryo or a weak spot in the grain or seed pericarp, but may devour the whole kernel during severe infestations (Pruthi & Singh, 1950). Furthermore, larvae have the capacity to feed in products with a moisture content of as low as 2% (Hinton, 1945). Under severe infestation, kernels are hollowed out and filled with frass and cast skins that completely deteriorate the quality, resulting in 100% loss (Ahmedani *et al.*, 2009). Infestations generate unpleasant odours and tastes, and impart a dirty appearance to the affected products (Khare *et al.*, 1974). These features have earned khapra beetle the reputation as a dirty feeder because it contaminates the product along with consumption (Stibick, 2009). Cast larval skins may cause dermatitis, gastro-intestinal and respiratory hazards to people who handle heavily-infested grains in grain mills (Stibick, 2009). Bristly larval hairs that rub off and remain in grains can pose serious health risks if swallowed, particularly to children, who may subsequently develop health issues such as ulcerative colitis, vomiting and diarrhea (Pruthi & Singh, 1950; Stibick, 2009).

Khapra beetle infestations also lead to nutrient depletion in grains (Girish *et al.*, 1975; Prasad *et al.*, 1977; Stibick, 2009). At 75% infestation levels, total carbohydrates, sugars, crude fat, protein nitrogen and true protein contents have been reported to decrease significantly in cereal grains (wheat, maize, sorghum), with a corresponding increase in moisture, crude fibre and total proteins (Jood & Kapoor, 1993; Jood & Kapoor, 1994; Jood *et al.*, 1996). However, total nitrogen, non-protein nitrogen, and uric acid levels increased substantially at 25% and higher levels of infestation (Jood & Kapoor, 1993). Significant losses of vitamins (thiamine, riboflavin, and niacin) and minerals (calcium, phosphorus, iron, zinc, copper, and manganese) occur at infestation levels of 25% and above, with reduced starch content at 50% infestation (Jood *et al.*, 1992b; Jood & Kapoor, 1994). At 50% and above infestation levels, total lipids, phospholipids, galacto-lipids, and polar and non-polar lipids decrease significantly (Jood *et al.*, 1996).

Unlike most stored-product insects, larvae of khapra beetle can enter diapause. Diapause is a period of suppressed growth and development in insects (Danks, 1987). With facultative diapause, khapra beetle can survive under a wide variety of abiotic conditions, such as hot and dry environments (Banks, 1977). Alone or in combination, several factors can induce diapause, including low temperatures, larval crowding, high relative humidity, the presence of fecal pellets, and low quality diet (Burges, 1963; Nair & Desai, 1972). This unusual diapause contributes immensely to spread of khapra beetle populations across the globe, because diapausing larvae can survive long periods of starvation in shipping containers and vessels carrying cargo between countries (Athanassiou *et al.*, 2019).

Little is known about the factors that lead to the termination of larval diapause in khapra beetle. Typically, diapause termination is achieved through high temperatures, whereas, paradoxically, food availability has been reported to be far less important or play a role only under conditions of isolation (Burges, 1962a; Nair & Desai, 1973). Burges (1963) reported that fresh food will terminate diapause in a subset of larvae with the remainder staying in diapause and becoming active for brief foraging excursions. Hence, the relationship between diet quality and diapause termination requires further study to better understand diapause in khapra beetle. Furthermore, larvae may stay in diapause for periods exceeding four years (Athanassiou *et al.*, 2019). However, little is known about how the duration in diapause influences diapause termination.

The major goal of the current study was to evaluate the effects of diet quality and age of culture (as a proxy for duration in diapause) on diapause termination of khapra beetle larvae. To achieve this objective, larvae that had been in diapause for different periods of time were fed diets of different nutritional quality to assess diapause termination and mortality during a six-month observational period.

2.3 Materials and methods

2.3.1 Insect's origin and rearing

The beetles for the study originated from collections made in a Pakistani grain market in 2011. From these collections, a colony of khapra beetle was established in the Grain Research Laboratory, Training and Storage Management Cell (Department of Entomology, Faculty of Agriculture, University of Agriculture) in Faisalabad, Punjab, Pakistan (Wilches *et al.*, 2017). In 2013, larvae from this colony were imported into Canada (Canadian Food Inspection Agency Permit # P-2013-01610) and used to establish colonies housed in Agriculture and Agri-Food Canada's quarantine facility at Lethbridge, Alberta. The colonies were reared in 4-litre jars containing 200 g diet (Wilches *et al.*, 2017). The rearing media comprised 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) in the ratio of 47.5, 17.5, 17.5 and 17.5%, respectively, on a weight basis (Wilches *et al.*, 2017). The conditions of rearing were 16:8 hour (light:dark) photoperiod at 30 ± 2 °C and 20 ± 10 % RH (all values are means ± SEM).

2.3.2 Experimental protocol

Larvae were sieved from the spent diet of colony jars that had been started 3, 10 or 14 months previously - no fresh diet had been added to these jars in the interim. Under ideal conditions and at 30 °C, egg-to-adult development normally takes about four to six weeks with adults living for about 5-10 days (Hadaway, 1956; Lindgren & Vincent, 1959). Because no live adults were observed at the time of sieving, it was assumed that larvae had exhausted the nutritional quality of the diet and had entered diapause. I subsequently distinguish among these larvae by the term “age of culture”, which I use as a proxy for being in diapause for different periods of time.

Five combinations of fresh diet (FD) and spent diet (SD) were chosen for the experiment. Fresh diet was the same diet as that used for rearing. Spent diet (SD) was recovered from a colony jar established 10 months previously with fresh diet. Specifically, it comprised the fine-textured material obtained by sieving the contents of the colony jar through a 250 µm sieve (U.S. standard sieve # 60 mesh) to remove larvae, dead adults, cast larval skins and frass. Fresh diet and spent diet represented diets of high and low nutritional quality, respectively. Mixtures of these diets (based on weight) spanned a range of intermediate nutritional qualities. Because of differences in average density, 5 g of spent and fresh diet corresponded to volumes of 6.7 and 9.0 mL, respectively. The diets used, as a percentage of fresh diet were: 100 (0% SD), 75 (25% SD), 50 (50% SD), 25 (75% SD) and 0 (100% SD).

For each combination of age of culture (3, 10 or 14 months) and diet (100, 75, 50, 25 and 0% FD), I set up 10 replicates. Each replicate comprised a glass Petri-dish (100 mm dia. x 15 mm ht.) containing 10 larvae in 2 g of diet (= 150 Petri dishes; 1500 larvae in total). This larval density was chosen because higher densities reportedly can induce diapause (Burges, 1963;

Wilches *et al*., 2017). The larvae were monitored for diapause termination at constant rearing conditions (30 ± 2 °C and 20 ± 10 % RH, complete darkness) at different day intervals. The first observation was recorded on Day 10 after experimental set-up. Subsequent observations were recorded every three or four days until Day 56, every14 days until Day 128, and then every 30 days until Day 188.

2.3.3 Diet analysis

To compare their nutritional qualities, samples of spent and fresh diet were sent for analysis to Dairy One, Inc., a feed testing facility in Ithaca, New York, United States. Spent diet was frozen to ensure the samples did not contain live khapra beetle before leaving quarantine. However, freezing might potentially alter diet quality. Hence, there was a need to compare diet quality of fresh diet (frozen vs. non-frozen). Assessment of all the diet parameters was performed in three replicates for each sample; (1) spent diet (frozen at -20 °C for two weeks); (2) fresh diet (frozen at -20 °C for two weeks); (3) fresh diet (not frozen, i.e., freshly prepared). The diets were analyzed for the following: moisture, pH, dry matter, crude protein, crude fat, ash, and fibre.

2.3.4 Statistical analysis

Data for diapause termination were analyzed with a two-way repeated measures analysis of variance. Individual Petri-dishes were treated as subjects and a full factorial ANOVA model was fit for the response variable; i.e., diapause termination (those larvae that broke diapause and became adults). Diet quality, age of culture, observation time (hereafter ‘time’) and their two-and-three-way-interactions were included as fixed effects. Subject was nested within age of culture and diet quality as a random effect. The remaining response variables, larvae remaining in diapause after 188 days and mortality, were analyzed separately with a two-way analysis of variance (Unlike with diapause termination, these response variables were measured only at the end of the study; therefore, repeated measures analysis was unsuitable). Log transformation, "log (x + 1)", was necessary to normalize the data and to account for zero counts (Zar, 1999; Arthur *et al.*, 2019).

The Kaplan-Meier survival analysis is one of the best ways to estimate the proportion of subjects who live for a certain amount of time after the application of a treatment (Fields *et al.*, 2010). Hence, this procedure was used to estimate the mean pupation time. Kaplan-Meier survival analysis was conducted using SigmaPlot (version 13.0).

Repeated measures ANOVA and two-way ANOVA were carried out in JMP 11 (SAS Institute Inc., 2013) and R (version 3.5.1) in RStudio (R Core Team, 2013), respectively.

2.4 Results

2.4.1 Diet composition

There was an increase in mean percent moisture, crude protein, fibre, and ash contents in the spent diet compared to fresh diet (Table 2.1). Dry matter, fat, and pH content of the spent diet samples decreased as compared to fresh diet. The freezing of samples before the analysis did not affect the outcome of results in subsequent analysis.

2.4.2 Diapause termination

As the proportion of fresh diet decreased in the diet mixture, the larvae required longer time to break diapause (Fig. 2.1, Table 2.3). No larvae provided with 100% spent diet terminated diapause. Diapause termination was bimodal. Initially, a proportion of the population terminated diapause during the first 30 to 60 days, then there was a plateau with little diapause termination with intermittent moults and sporadic feeding, then another burst of diapause termination in the last 30 days before the experiment was terminated (Fig. 2.1). This second peak of diapause termination coincided with the phase of the experiments in which the observation interval was prolonged (Fig. 2.1).

Overall, after 188 days, the total diapause termination on different diet qualities increased with greater percentage of fresh diet; i.e., 87, 76, 62, 42, and 0% for 100, 75, 50, 25, 0% fresh diet, respectively (averaged across culture ages). The two-way repeated measures ANOVA detected significant differences ($P < 0.05$) for the main effects and its interactions (Table 2.2).

The effect of age of culture on diapause termination was complex. The larvae extracted from the 10-month old culture required less time to break diapause than the larvae from the cultures aged 3 and 14 months (Table 2.2). The mean percentage diapause termination for larvae decreased with increased age of the culture; i.e., 57, 55, and 48% for 3, 10, and 14 months, respectively (averaged across diet qualities) (Fig. 2.1). No diapausing larvae terminated diapause on 0% fresh diet, regardless of culture age. Hence, this latter treatment was excluded from the analysis.

2.4.3 Maintenance of diapause after 188 days

More larvae tended to remain in diapause as the proportion of fresh diet decreased in the diet mixture (Fig. 2.2). By the end of the study, the percentage of larvae remaining in diapause on the different diets was 4, 14, 25, 26 and 0; 100, 75, 50, 25 and 0% FD respectively (averaged across age of culture). When averaged across diets, the percentage of larvae remaining in diapause was 24, 11, and 14% for cultures aged 3, 10, and 14 months, respectively. Age of culture ($F = 18.28$, df = 2, 149; $P < 0.001$), diet quality ($F = 28.63$, df = 4, 149; $P < 0.001$) and their interaction ($F = 5.87$, df = 8, 149; $P < 0.001$) each had a significant effect ($P < 0.05$) on the percentage of larvae remaining in diapause.

2.4.4 Mortality

Larvae also were less likely to survive on diets of lower quality (Fig. 2.3). On the 0% fresh diet, larvae were observed to noticeably lose body mass. By the end of the study, percentage larvae mortality on the different diets was 8, 10, 13, 32 and 88; 100, 75, 50, 25 and 0% FD, respectively (averaged across age of culture). Larvae from older cultures were most affected by low quality diets. On the 0% fresh diet, percentage larval mortality was 67, 97 and 100 for cultures aged 3, 10 and 14 months, respectively (Fig. 2.3). For results combined across all diets, percentage larval mortality was 20, 33 and 38 for cultures aged 3, 10 and 14 months, respectively. A significant effect ($P < 0.05$) on mortality was detected for age of culture (F = 22.00, df = 2, 149; $P < 0.001$) and diet quality (F = 111.02, df = 4,149; $P < 0.001$), but not for their interaction (F = 1.32, df = 8, 149; P = 0.23).

2.5 Discussion

There were substantial increases in the contents of moisture, crude protein, fibre and ash in the spent diet compared to fresh non-infested diet. A maximum increase in crude protein content of 51% was observed. This result is in accordance with previous reports of high protein content in grain samples infested with khapra beetle (Girish *et al.*, 1975; Jood & Kapoor, 1993; Ahmedani *et al.*, 2009). However, the presence of high crude protein levels in infested grain is not intuitive, because feeding by khapra beetle larvae on the protein-containing germ portion of the grain would presumably lower protein concentration (Ahmedani *et al.*, 2009). Therefore, an inverse relationship should have existed between the level of infestation and the protein content of diet (Ahmedani *et al.*, 2009; Ahmedani *et al.*, 2011). However, I agree with a number of other studies that attribute this discrepancy to the presence of non-digestible protein fragments of exuviae and body parts that were not removed from the spent diet during the sieving process

(Jood *et al.*, 1992a; Ahmedani *et al.*, 2009; Ahmedani *et al.*, 2011). The same explanation applies to increased fibre content in the spent diet; i.e., insect exoskeletons contain a significant amount of fibre (Kouřimská & Adámková, 2016). On the other hand, researchers also attribute increased protein levels to significant carbohydrate depletion and increased levels of total nitrogen and uric acid contents (Jood & Kapoor, 1993; Mebarkia *et al.*, 2010; Saad *et al.*, 2018). Furthermore, there was also a change in colour and pungent foul odour in the spent diet, which might reflect the presence of uric acid in the dietary sample. Significant increases in levels of moisture, ash, crude protein, crude fibre, non-protein nitrogen and uric acid have also been observed in stores of wheat that have been infested by insects for a long period of time (Samuels & Modgil, 1999). Therefore, Aja *et al.* (2004) suggest that when evaluating the effect of insect infestation on the protein content of stored grains, the gluten index should be evaluated as an index of protein quality (Ahmedani *et al.*, 2009).

I speculate that the bimodal pattern of diapause termination may reflect one or, more likely, a combination of factors. One of these factors could be changes in the frequency of disturbance during the observation period. Larvae were handled with forceps to determine their status every three or four days beginning on Day 10 until Day 56, thereafter every 14 days until Day 128, and then every 30 days until Day 188. In the closely-related species *Trogoderma variabile* Ballion (Coleoptera: Dermestidae), disturbance has been reported to induce diapause (Loschiavo, 1960). Potentially, disturbance may have the opposite effect in *T. granarium* with the higher frequency of disturbance during the early part of the study contributing to the first peak of diapause termination. A second factor could be the requirement for diapausing larvae to accumulate a minimum amount of energy (an energy threshold) before they can terminate diapause; i.e., pupate (Burges, 1963; Nair & Desai, 1973). Larvae with reserves just below this threshold (in shallow diapause) would be able to more quickly meet this shortfall than larvae much below this threshold (in deep diapause). The bimodal pattern observed in the current study may therefore reflect diapause termination by individuals in shallow (early termination) versus deep (late termination) diapause. Differences in energy reserves among individuals selected for the study could arise from different generational cohorts present in the same culture jar and (or) different sexes (Hadaway, 1956). A confounding factor would be the speed with which energy can be acquired by diapausing larvae, which could vary between individuals in different depths of diapause and (or) of different gender.

The relationship between the termination of larval diapause and diet quality has direct application for khapra beetle control programs. Adult khapra beetles do not fly (Hadaway, 1956). Thus, it is the movement of larvae, typically in contaminated shipping containers and vessels, that contributes to the spread of this pest into new regions (Athanassiou *et al.*, 2019). The enhanced tolerance of diapausing larvae to starvation, insecticides and extreme temperatures reduces the effectiveness of available control measures to compound the problem. Therefore knowledge of factors affecting diapause termination in khapra beetle is expected to assist in the development of more effective control programs. It is also significant for quarantine programs. As diapausing larvae could be a large proportion of khapra beetle landing in quarantine countries. Thus information on diapause termination will better predict the establishment of populations in quarantine countries.

I recommend that future studies examine in more detail, the bimodal pattern of diapause termination observed in the current study. Doing so should shed more light on the complex interaction between diapause termination and depth of diapause, and confounding factors of diet quality and potentially of gender.

Table 2.1 Comparison for spent-and-frozen, fresh-and-frozen and fresh-and-not-frozen khapra diet. Spent diet was diet that had undergone khapra beetle larvae infestation for ten months. Values are means ± SE of three replicate samples.

Diet type	Frozen	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Fibre (%)	pH
Spent diet	Yes	14.9 ± 0.1	88.0 ± 0.2	4.8 ± 0.1	15.8 ± 0.1	5.0 ± 0.1	4.6 ± 0.0
Fresh diet	Yes	8.1 ± 0.0	36.7 ± 0.1	8.6 ± 0.2	6.6 ± 0.1	1.3 ± 0.2	5.8 ± 0.0
Fresh diet	No	7.9 ± 0.0	36.6 ± 0.0	7.7 ± 0.8	6.7 ± 0.1	1.3 ± 0.2	5.9 ± 0.0

Table 2.2 Repeated measures analysis of variance for main effects and their interactions for diapause termination of diapausing khapra beetle larvae provided with diets varying in nutritional quality (ndf = numerator degrees of freedom, ddf = denominator degrees of freedom).

Source	**ndf**	**ddf**	**F**	***P***
Age of culture	2	108	24.91	<0.0001
Diet quality	3	108	81.19	<0.0001
Age of culture × Diet quality	6	108	4.55	0.0004
Time	20	2160	537.71	<0.0001
Age of culture × Time	40	2160	14.36	<0.0001
Diet quality × Time	60	2160	20.65	<0.0001
Age of culture × Diet quality × Time	120	2160	4.43	<0.0001

Table 2.3 Effect of diet quality (mixtures of fresh and spent) and age of culture (duration in diapause) on pupation time (diapause termination) based on Kaplan-Meier survival analysis (*no analysis was conducted because there was no diapause termination).

Age of culture (months)	**Pupation time (days; mean ± SE)**					**Gehan-Breslow statistic**	**df**	***P***
	Diet quality (% fresh diet)							
	100	**75**	**50**	**25**	**0***			
3	84 ± 7^{aB}	136 ± 6^{bB}	159 ± 5^{bB}	143 ± 7^{bA}	-	76.67	3	< 0.001
10	40 ± 5^{aA}	119 ± 7^{bA}	107 ± 8^{bA}	151 ± 7^{cB}	-	135.32	3	< 0.001
14	97 ± 8^{aC}	143 ± 6^{bB}	143 ± 6^{bB}	168 ± 4^{cB}	-	67.70	3	< 0.001
Gehan-Breslow statistic	29.794	11.76	13.46	12.64				
df	2	2	2	2				
P	0.001	0.003	0.001	0.002				

Mean pupation time ± SE (in days) of diapausing khapra beetle larvae on five diet qualities obtained from three different colony ages based on Kaplan-Meier survival analysis. Within columns, means followed by the same uppercase letters are not significantly different; within rows, means followed by the same lowercase letters are not significantly different (*P* = 0.05), multiple comparison test Holm-Sidak method.

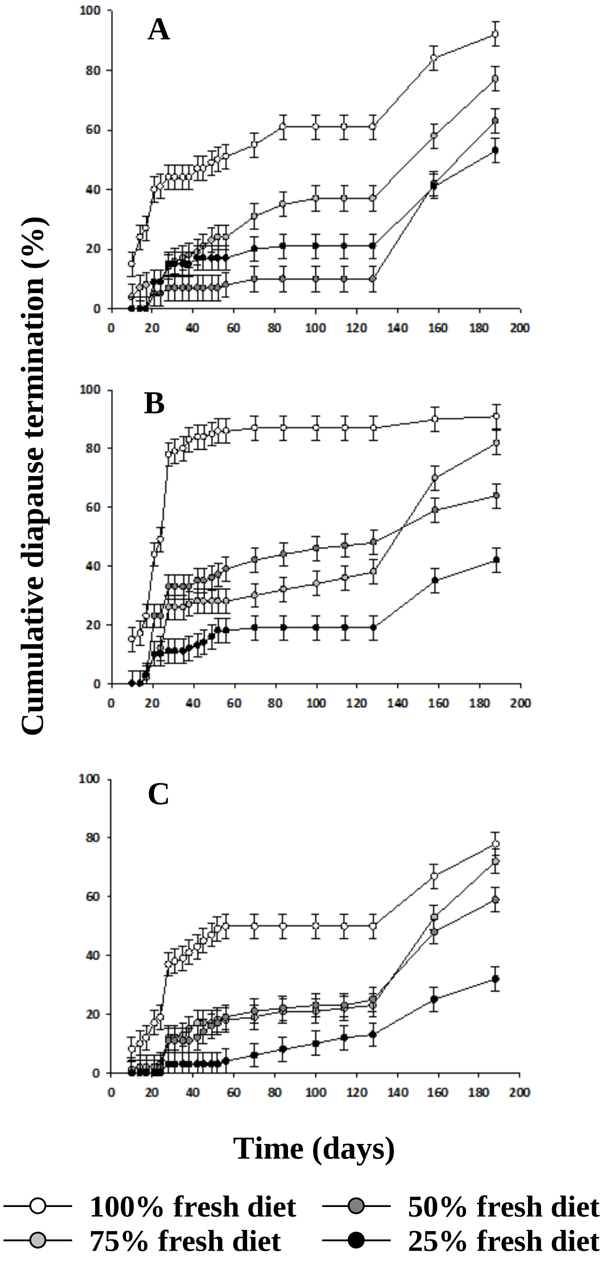

Figure 2.1 Effect of diet quality and age of culture on cumulative diapause termination of **A)** three month, **B)** ten month, **C)** Fourteen month old khapra beetle colony larvae (10 larvae per Petri dish, 10 replicate Petri dishes per combination of fresh diet and age of culture; error bars: Least Square Means ± SEM) when reared on different combinations of fresh and spent diets.

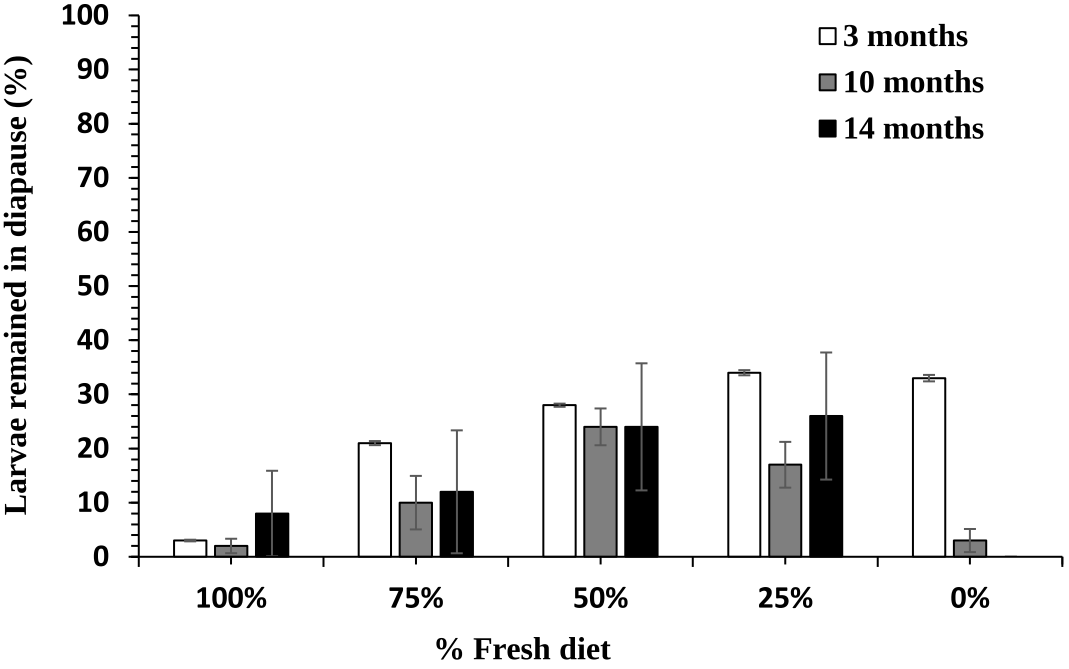

Figure 2.2 Effect of diet quality and age of culture on larvae that remained in diapause after 188 days (*n* = 10, replicates = 10, error bars: ± SE) when reared on different combinations of fresh and spent diets.

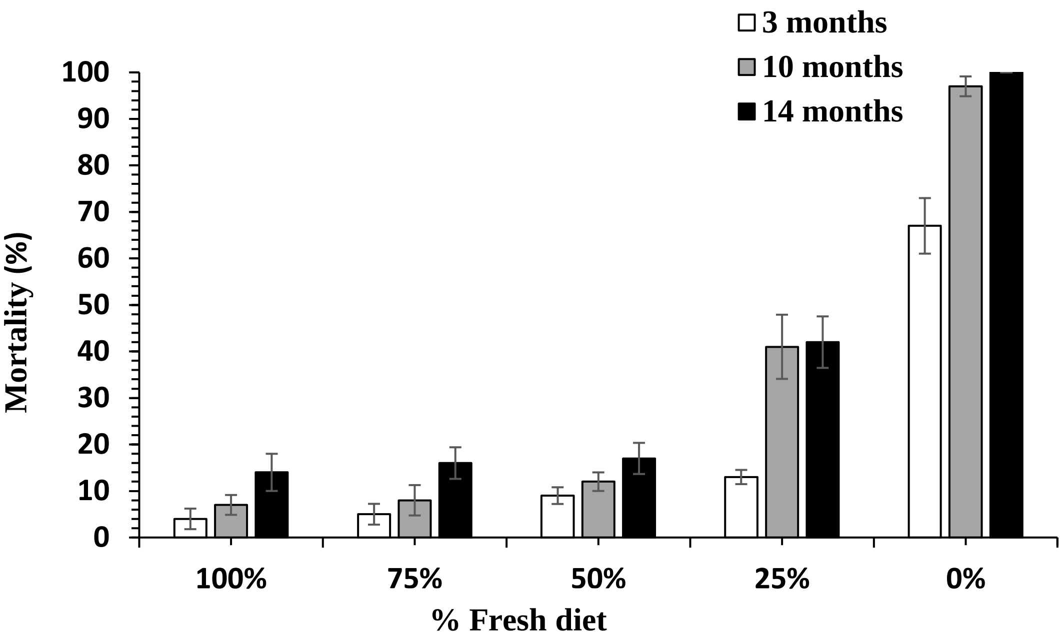

Figure 2.3 Effect of diet qualities and age of culture on larval mortality of diapausing khapra beetle larvae (*n* = 10, replicates = 10, error bars: ± SE) when reared on different combinations of fresh and spent diets.

CHAPTER 3: CROSS TOLERANCE TO DESICCATION AND COLD IN KHAPRA BEETLE, *Trogoderma granarium*

3.1 Abstract

Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), is unusual in two key respects. First, they are among the most cold-hardy of stored-product insect pests even though they originate in hot and dry regions of the Indian sub-continent. Second, their larvae can enter into a diapause state to survive harsh environmental conditions. In the current study, I examined whether these two phenomena might be related; i.e., cross-tolerance. Cross-tolerance is the tolerance to one ecological stress when induced by a separate stress. To investigate this, khapra beetle larvae were reared at different relative humidities (3, 28, 49 and 79%) either in non-diapausing or diapausing conditions. Then the cold-tolerance of larvae was estimated by measuring mortality after different durations at −10 °C. For non-diapausing larvae, relative humidity had little effect on cold tolerance with the lethal time to 50% mortality (LT_{50}) occurring between 2-4 days. For diapausing larvae, cold tolerance increased with greater desiccation stress with LT_{50}'s of 5, 7, 10 and 18 days at 79, 49, 28 and 3% RH respectively. This suggests that the physiological mechanisms that protect diapausing larvae from desiccation may also increase cold-tolerance, even though these insects may rarely be exposed to low temperatures in their typical habitats.

3.2 Introduction

Desiccation is a significant environmental stress that confronts overwintering insects. Among desiccation and numerous other environmental stressors encountered by insects, cold and desiccation stress are particularly believed to be governed by overlapping sets of regulatory and mechanistic processes (Sinclair *et al.*, 2013). Several studies report that cold and desiccation tolerance share many common traits such that the two stressors have a degree of cross-tolerance (Ramlov & Lee, 2000; Bayley *et al.*, 2001; Sinclair *et al.*, 2013; Terhzaz *et al.*, 2015). Solutes in the hemolymph and habitat selection play a dual role by conferring protection to both desiccation and cold (Ring & Danks, 1998). These protective mechanisms have been jointly referred to as "cross-tolerance" by Sinclair *et al.* (2013). Other adaptations to cold and desiccation stress include signalling pathways ("cross-talk") and responses at cellular, molecular and biochemical levels (Sinclair *et al.*, 2013). However, possible mechanisms for such inter-linked responses remain poorly understood (Sinclair *et al.*, 2013; Terhzaz *et al.*, 2015).

A common response to cellular dehydration - a primary mode of damage due to desiccation or freezing - is the production of low molecular weight compounds called cryoprotectants (Ring, 1994; Ring & Danks, 1998). These cryoprotectants are generated as a result of gene expression whose functions overlaps to confer protection against desiccation and cold-related injury. A few of these genes are responsible for the production of antifreeze proteins and trehalose that help preserve protein and membrane structure, thereby improving tolerance to desiccation or cold (Sinclair *et al.*, 2013). However, in some cases, increased cold and desiccation tolerance might be associated with diapause (Denlinger, 1991; Danks, 2000), but the relationship is unclear and remains controversial (Denlinger, 1991; Pullin, 1996). Moreover, adaptations that allow these insects to resist cold and desiccation may be coincidental with adverse environmental conditions, and may not be controlled by diapause alone (Danks, 2000). Therefore, species of tropical origin, with diapause in their life cycle, may be important model organisms for deciphering the interaction between diapause, cold and desiccation.

Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), is a pest of stored products in hot, dry countries stretching from Egypt to India; it is a quarantine insect for much of the rest of the world (Athanassiou *et al.*, 2019). It has facultative larval diapause that increases its resistance to insecticides and extreme temperatures, making it particularly difficult to eradicate. Although it is only common in hot and arid countries, it is one of the most cold-tolerant of stored product insects (Fields, 1992; Wilches *et al.*, 2017). Diapause and cold acclimation enhance its cold-hardiness, which requires exposure of 347 days at −10 °C to kill all individuals (Wilches *et al.*, 2017). Other insect species that are extremely cold hardy are mostly from temperate polar regions (Turnock & Fields, 2005; Hayward *et al.*, 2007). Hence, there is speculation that khapra beetle's remarkable cold-hardiness may be due to cross-tolerance with desiccation (Sinclair *et al.*, 2013); i.e., because of its adaptation to dry and hot climates, it could be pre-adapted to cold environments (Wilches *et al.*, 2017). Thus, additional experiments are necessary to test the notion of cross-tolerance and clarify the complex relationship between diapause, cold-hardiness and desiccation.

The major goal of the current study was to assess whether desiccation tolerance enhances cold tolerance in diapausing and non-diapausing khapra beetle larvae. The hypotheses of the study were that (1) diapausing larvae have an enhanced capacity to survive exposures at low temperatures compared to non-diapausing larvae, (2) diapause and desiccation enhance the cold-tolerance levels of larvae synergistically, with non-diapausing larvae exhibiting limited cold-hardening capacity.

3.3 Materials and Methods

3.3.1 Insect's origin and rearing

The beetles for the study originated from collections made in a Pakistani grain market in 2011. From these collections, a colony of khapra beetle was established in the Grain Research Laboratory, Training and Storage Management Cell (Department of Entomology, Faculty of

Agriculture, University of Agriculture) in Faisalabad, Punjab, Pakistan (Wilches *et al.*, 2017). In 2013, larvae from this colony were imported into Canada (Canadian Food Inspection Agency Permit # P-2013-01610) and used to establish colonies housed in Agriculture and Agri-Food Canada's quarantine facility at Lethbridge, Alberta. The colonies were reared in 4-litre jars containing 200 g diet (Wilches *et al.*, 2017). The rearing media comprised 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) in the ratio of 47.5, 17.5, 17.5 and 17.5% respectively, on a weight basis (Wilches *et al.*, 2017). The conditions of rearing were 16:8 hour (light:dark) photoperiod at 30 ± 2 °C and 20 ± 10 % RH (all values are means ± SEM).

Khapra beetle diapause as larvae, so only this particular stage was evaluated in these experiments. The eggs were obtained according to a procedure described by Yinon (1965) with slight modifications. Thirty adult beetles were placed in a translucent cylindrical polypropylene container (Nalgene™) with a screw-cap closure. The original bottom of the container was removed and fitted with a fine plastic mesh screen in such a way that the screen allowed for the passage of eggs but not the adult males or females. The containers were placed on top of a cardboard paper rested inside the glass Petri dish and maintained at 30 ± 2 °C and 20 ± 10 % RH, to facilitate egg laying. Egg hatching was monitored for 10-15 days and newly hatched, first-instar larvae were used for subsequent experiments.

3.3.2 Desiccation

Desiccators were set up to obtain four different relative humidities using saturated salt solutions, established according to Winston & Bates (1960). The established relative humidities were verified with the help of HOBO UX100, temperature/relative humidity data loggers (Onset

Computer Corporation, Bourne, MA). Larvae were reared in glass Petri-dishes placed above the saturated salt solutions used to generate 3% RH (CaSO4), 28% RH (CH_3COOK), 49% RH ($Mg(NO_3)_2$), and 79% RH (NaCl). The desiccators were placed at 30 ± 2 °C in a 16:8 hour (light:dark) photoperiod light cycle. Larvae were reared on diet held at the various relative humidities in desiccators for two weeks prior to use. Pre-tests showed that the moisture content of the diet reached equilibrium with the relative humidity by the end of two weeks. The moisture content of the diet at different RH were 3.7, 5.1, 7.1, and 12.1% at 3, 28, 49 and 79% RH, respectively. Because cold-tolerance was a key variable in this study, it was necessary to develop a method to reliably source diapausing larvae that didn't involve temperature treatment. Therefore, based on previous studies (Wilches *et al.*, 2017) and additional experiments (Appendix 1), non-diapausing larvae (NDL) were obtained by low-density rearing (25 larvae/6 g) for 18 days after hatch, and diapausing larvae (DL) were obtained by rearing at densities greater than 220 larvae/6 g for 45 days after hatch.

3.3.3 Assessment of larval cold-tolerance

Diapausing and non-diapausing larvae from the different desiccators were placed in polypropylene vials (10 cm height x 2.8 cm diameter, 20 larvae vial) with 3 g of diet previously equilibrated to the relative humidity of the corresponding desiccator. The vials were placed in a freezer at a constant temperature of −10 °C (Scientemp™, Adrian, Michigan, United States) for different periods of exposure based on findings reported by Wilches *et al.* (2017). For non-diapausing larvae, these periods were 0 (control), 1, 2, 3, 4, 5, 7 and 14 days. For diapausing larvae, these durations were 0 (control), 3, 7, 14, 21 and 28 days. Upon removal from −10 °C, vials were held at 30 °C. After five days at 30 °C, larval mortality was assessed by observation of movement after tactile stimulation. After 15 days at 30 °C, diapause termination (pupation) was assessed to determine the proportion of larvae that had survived to become adults. For each combination of relative humidity, period of exposure to -10 °C and larval status (non-diapausing larvae and diapausing larvae), we used three replicate vials; i.e., non-diapausing larvae = 32 total vials; diapausing larvae = 24 total vials.

3.3.4 Statistical analyses

At a given temperature, a plot of time versus percent mortality has a sigmoidal shape; it is transformed into a linear relationship with probit analysis to estimate the lethal times. Probit analysis with logarithmic transformation was conducted separately for non-diapausing and diapausing larvae to estimate the lethal times (LT_{50}, LT_{95}, LT_{99}) (Robertson *et al.*, 2017). Probit analysis was carried out using PoloPlus 2.0, LeOra Software (Finney, 1971).

3.4 Results

3.4.1 Cold-tolerance of larvae following desiccation

Estimated lethal times (LT_{50}, LT_{95}, LT_{99}) for diapausing and non-diapausing larvae reared at different relative humidities and then exposed to −10 °C are summarized in Table 3.1. For each estimate, the χ^2 goodness-of-fit statistic vs. df showed that the probit model adequately fit the data (Table 3.1; $P > 0.05$). For diapausing larvae, being reared under drier conditions enhanced their cold-hardiness (Fig. 3.1B); i.e., estimated LT_{50} values of ca. 5, 7, 10 and 18 days at 79, 49, 28 and 3% RH respectively (Table 3.1). For non-diapausing larvae, the relative humidity of rearing condition did not affect cold-hardiness (Fig. 3.1A); i.e., estimated LT_{50} of ca. 3, 2, 2, and 4 days at 3, 28, 49, and 79% RH, respectively (Table 3.1).

3.4.2 Diapause termination post cold exposure

Non-diapausing and diapausing larvae that survived cold exposure and became pupae or adults after 15 days at 30 °C were considered to have terminated diapause. The percentage of diapause termination for non-diapausing larvae that were never exposed to -10 °C (controls) was high; i.e., 100, 100, 83 and 77 for larvae reared at 28, 49, 79, and 3% RH, respectively (Fig. 3.1C). In contrast, the percentage of diapause termination for non-diapausing larvae exposed to -10 °C for even 1 day was < 30 % (Fig. 3.1C). As expected, the diapausing larvae had a low percent diapause termination in the control group (3, 8, 10, and 13% reared at 79, 28, 49, and 3% RH, respectively, Fig 3.1D). Diapause termination remained low for all exposures.

3.5 Discussion

Results of the current study provide evidence of cross-tolerance in khapra beetle whereby the mechanisms that confer tolerance to desiccation stress also confer tolerance to cold stress (Sinclair *et al.*, 2013). Diapausing larvae reared under conditions of lower relative humidity (higher levels of desiccation stress) exhibited higher levels of cold-hardiness (Table 3.1, Fig. 3.1B). This finding agrees with previous reports that desiccation promotes cold-hardiness, especially in insects from temperate polar regions (Ring, 1994; Holmstrup & Sømme, 1998; Ring & Danks, 1998; Bayley *et al.*, 2001; Benoit *et al.*, 2009; Levis *et al.*, 2012; Sinclair *et al.*, 2013) but also in some tropical diapausing insects (Kostal *et al.*, 1998). For non-diapausing larvae, however, the cold-tolerance of larvae reared under different relative humidities was largely similar (Table 3.1, Fig. 3.1A). Further studies are needed to clarify this discrepancy.

Desiccation can lead to the upregulation of substances (sugars, polyols or amino acids) that act as cryoprotectants to enhance cold tolerance (Ring, 1994; Ring & Danks, 1998). One possible explanation of my results could be that non-diapausing larvae reared under desiccation stress produce such substances, but to a lesser extent or of a different composition than substances produced by diapausing larvae reared under similar conditions. The need for desiccation tolerance is much greater for insects in diapause, during which water is lost that cannot be replaced by feeding (Danks, 1987). Given that khapra larvae can survive in diapause for years (Athanassiou *et al.*, 2019), it is reasonable to expect that the substances produced by diapausing larvae would confer a greater level of tolerance to desiccation stress (and associated cross-tolerance to cold stress) than the substances produced by non-diapausing larvae.

Future work could measure substances (sugars, polyols or amino acids) that are commonly associated with desiccation and cold tolerance to see if these are affected by desiccation stress, cold acclimation or diapause. Future research should also consider if diapause and cold acclimation increase the desiccation tolerance of khapra beetle. Only a few studies demonstrate that the mechanisms which enhance survival to desiccation and heat are identical in their physiological responses (Hinton, 1960; Watanabe *et al.*, 2002; Benoit *et al.*, 2009). Because khapra beetle larvae are more tolerant to heat when in diapause (Wilches *et al.*, 2019), the effects of desiccation on heat tolerance would also be of interest. Finally, the estimation of the durations needed to control khapra beetle with low temperature (Wilches *et al.*, 2017) may need to be increased for insects exposed to dry conditions.

Table 3.1 Lethal time (LT in days; plus 95% confidence interval in parentheses) to achieve 50, 95, and 99% mortality of the population of non-diapausing and diapausing khapra beetle larvae at −10 °C reared over a range of relative humidities.

Relative humidity (%)	**N**	**Slope ± SE[a]**	**LT$_{50}$ (days, 95% CL)**	**LT$_{95}$ (days, 95% CL)**	**LT$_{99}$ (days, 95% CL)**	**Intercept ± SE[b]**	**χ^2 (df)**
Non-diapausing larvae							
3	8	2.39 ± 0.30	3.4 (2.8 – 4.1)	16.8 (12.5 – 26.5)	32.4 (21.5 – 62.3)	-1.27 ± 0.21	6.92 (19)
28	8	2.40 ± 0.25	2.1 (1.7 – 2.4)	10.0 (7.9 – 14.1)	19.3 (13.8 – 31.8)	-0.75 ± 0.13	8.31 (19)
49	8	2.76 ± 0.27	2.2 (1.9 – 2.5)	8.6 (7.0 – 11.4)	15.1 (11.4 – 22.8)	-0.92 ± 0.14	14.18 (19)
79	8	2.61 ± 0.38	3.7 (2.9 - 4.5)	15.8 (11.8 – 25.4)	28.7 (19.2 – 57.9)	-1.48 ± 0.29	5.16 (19)
Diapausing larvae							
3	6	2.10 ± 0.52	18.0 (11.6 – 24.2)	109.5 (55.5 – 1639.4)	231.7 (89.3 – 11147.0)	-2.62 ± 0.68	17.16 (13)
28	6	1.75 ± 0.23	9.7 (7.1 – 12.6)	83.9 (47.8 – 251.4)	205.5 (94.5 – 965.2)	-1.72 ± 0.25	18.43 (13)
49	6	1.85 ± 0.24	6.6 (4.6 – 8.5)	51.1 (32.4 – 117.8)	119.5 (62.7 – 404.8)	-1.51 ± 0.24	17.59 (13)
79	6	1.91 ± 0.27	4.8 (3.3 – 6.2)	34.9 (24.8 – 60.0)	79.1 (48.4 – 180.8)	-1.30 ± 0.27	8.48 (13)

The χ^2 goodness-of-fit statistic vs. df for non-diapausing larvae and diapausing larvae indicates that the probit model fits the data adequately ($P > 0.05$).

[a] Slope and intercept of the probit model regression line.

[b] $n = 20$ larvae per vial in three replicates, per duration

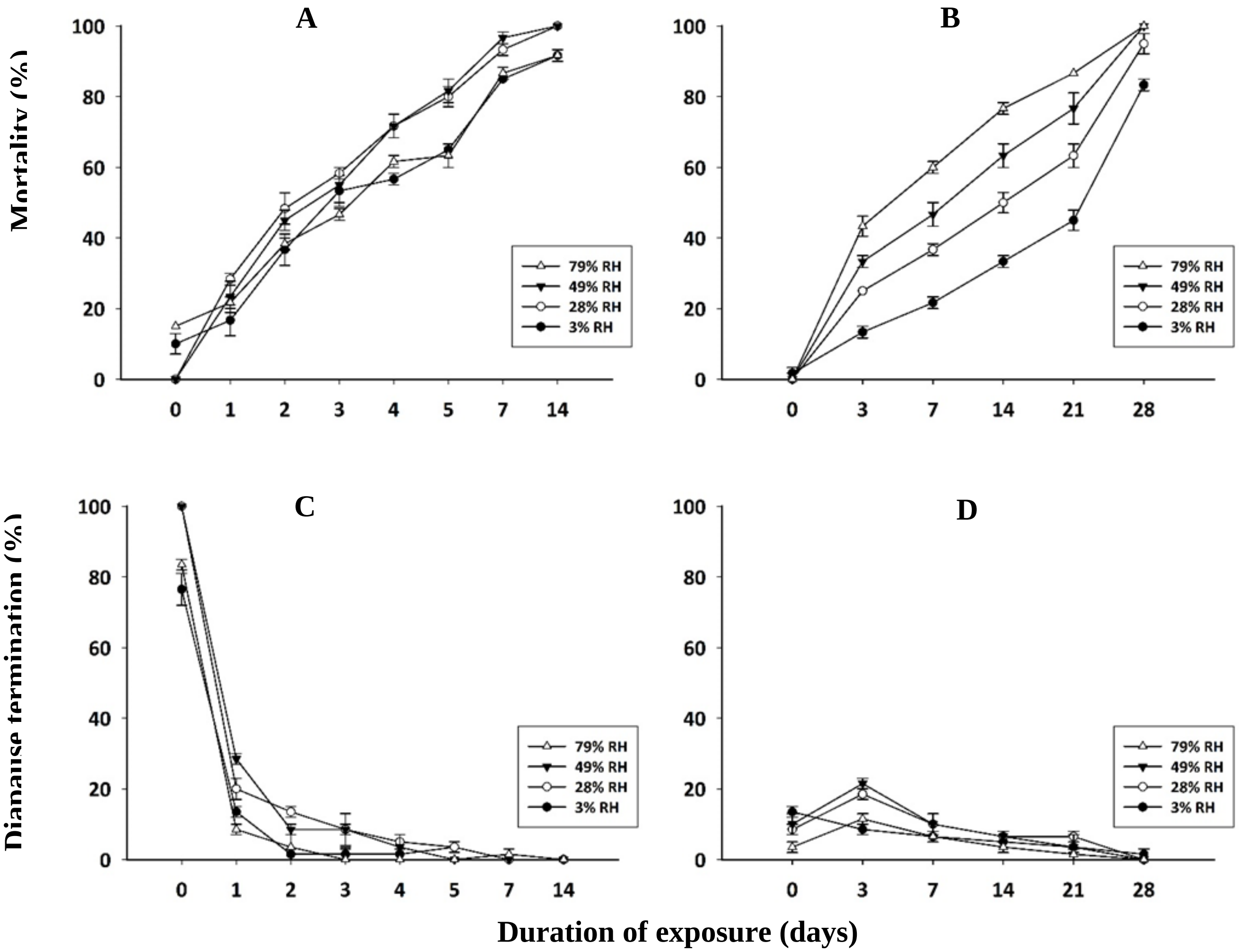

Figure 3.1 Fate of larvae acclimated to one of four different relative humidities and then exposed to −10 °C for periods of up to 14 (**A & C** = non-diapausing) or 28 (**B** & **D** = diapausing) days. Larvae were subsequently held at 30 °C for a further 15 days and then their status recorded. Values are means ± SE of three replicate vials, each containing 20 larvae at the commencement of low-temperature exposure.

CHAPTER 4: GENERAL DISCUSSION

4.1 Summary

Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), is a pest of stored products and is considered a quarantine insect for much of the rest of the world (Eliopoulos, 2013). It is native to India, but is currently distributed in Asia, Africa, the Middle East, and a few European countries (Athanassiou *et al.*, 2019). Unlike most stored-product insects, it is capable of entering facultative larval diapause. This allows it to survive for several years as a diapausing larva with little to no food, and increases its resistance to insecticides and extreme temperatures, making it particularly difficult to eradicate. Several factors induce diapause in khapra beetle. Although such factors that induce diapause have been well studied, very little is known on the factors that terminate it. Hence, the current thesis seeks to address and add to the general body of knowledge on key biological aspects (diapause induction and termination) of khapra beetle.

In Chapter 1, I reviewed the literature on general aspects of insect diapause induction and termination, particularly in Dermestidae. Because of khapra beetle's association with diapause, I also reviewed the concept of cross-tolerance between cold and desiccation in insects and different physiological responses associated with it.

In Chapter 2, I investigated the effects of diet quality and age of culture on diapause termination in khapra beetle larvae. Age of culture refers to the duration for which a colony has been maintained on the same diet. Colonies of older duration have larvae that have been in diapause for a longer period of time. The results showed that diapause termination was affected by the quality of diet and age of culture. The diapausing larvae were able to feed and resume development and complete their life cycle on sub-optimal diet, which highlights the ability of this pest to survive under harsh conditions (Nair & Desai, 1973). When provided with fresh diet, termination of larval diapause exhibited a bimodal pattern. Most larvae broke diapause in the first 30-60 days, but a portion of the population continued to feed sporadically with additional moults and accumulate nutrients for few days or months before breaking diapause. Diapause termination started to level off after 56-60 days with the highest levels of diapause termination on diets of the highest quality. Diapausing larvae can survive for long periods in empty shipping containers or storage bins. These results illustrate how filling these containers or bins with grain can induce diapause termination to enable the subsequent growth of the khapra beetle population.

In Chapter 3, I present the results of two experiments. First, I investigated the effects of larval crowding on diapause induction. Second, I assessed whether desiccation enhances the cold tolerance of non-diapausing and diapausing khapra beetle larvae. Results from the diapause induction experiment showed that khapra beetle larvae entered diapause at densities exceeding 38 larvae/g of diet, but generally did not enter diapause when reared in isolation. These results were similar to those of Wilches *et al.* (2017) for khapra beetle and for other species of dermestids (Burges, 1963; Nair & Desai, 1972; Barak & Burkholder, 1977b). Previous researchers have suggested that diapause induction of khapra beetle larvae at high densities may reflect a decline in diet quality, an increase in fecal waste and associated compounds (e.g., linoleic acids), or a combination of both (Burges, 1963; Stanic *et al.*, 1963; Karnavar & Nair, 1969b; Stanic & Shulov, 1969).

In the second experiment of Chapter 3, larvae were reared at different relative humidities (3, 28, 49 and 79%) either in non-diapausing (25 larvae/6 g diet) or diapausing conditions (<220 larvae/6 g diet). Then, survival was assessed at -10 °C. For non-diapausing larvae, rearing conditions had little effect on cold tolerance with the lethal time to 50% mortality (LT_{50}) occurring between 2-4 days. For diapausing larvae, cold tolerance increased with greater desiccation stress with LT_{50}'s of 5, 7, 10 and 18 days at 79, 49, 28 and 3% RH respectively. This finding supported the hypothesis that cold and desiccation stress are linked and share many common traits with a degree of cross-tolerance between them (Sinclair *et al.*, 2013). The finding is also in accordance with various studies where the role of desiccation in promoting the cold-hardiness had been observed, especially in insects from temperate polar regions (Ring, 1994; Holmstrup & Sømme, 1998; Ring & Danks, 1998; Bayley *et al.*, 2001; Benoit *et al.*, 2009; Levis *et al.*, 2012; Sinclair *et al.*, 2013) and also in few tropical diapausing insects (Kostal *et al.*, 1998).

4.2 Practical considerations and future directions

Future studies should test the effects and interaction of multiple factors (biotic and abiotic) in inducing diapause in khapra beetle, because one factor (e.g., density) in isolation may not be sufficient to induce diapause (Nair & Desai, 1972). In particular, future studies should identify cues for diapause induction and termination and develop methods to better distinguish between diapausing and non-diapausing individuals. Studies should also identify specific factors that induce diapause in warehouse and storage conditions, and artificially manipulate these factors to help enhance already established control methods.

In Chapter 2, I determined that the age of culture (duration in diapause) affected diapause termination. This assumed that the larvae sieved from these cultures of different age (3, 10, and 14 months) were in diapause. However, the results also showed that the age of culture (which I assumed to be a proxy for duration in diapause) on diapause termination was complex. Therefore, future studies should clearly identify and induce diapause for various lengths of time to better understand the influence of 'diapause depth' on 'diapause termination' in khapra beetle.

Results from Chapter 3 showed that desiccation enhanced the cold-tolerance of diapausing larvae. This relationship could be examined in more detail by measuring the differences in cryoprotectants (sugars, polyols, and amino acids) in both diapausing and non-diapausing larvae exposed to desiccation stress, as desiccation can lead to upregulation of substances (Ring, 1994; Ring & Danks, 1998). Because khapra beetle larvae are also heat tolerant, further studies could be carried out to test cross tolerance of khapra beetle to heat.

In conclusion, this thesis adds to the general body of knowledge on khapra beetle biology (diapause induction and termination, cold and desiccation tolerance). It also provided the first evidence of cross-tolerance in khapra beetle whereby the mechanisms that confer tolerance to desiccation stress also confer tolerance to cold stress. This is particularly important for khapra beetle and has direct application for its control programs. This new information can help assist in the development of more effective physical control methods. The estimation of the durations needed to control khapra beetle infested shipments with low temperatures from hot and dry climates, where khapra beetle is known to occur, may need to be increased. Previous research on khapra beetle biology is scarce and most such studies were published nearly 40 years ago (Athanassiou *et al.*, 2019). Taking into consideration the elements of larval diapause and cross-tolerance phenomenon while employing contemporary management strategies will be essential to attain maximum levels of control.

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APPENDIX 1: INDUCTION OF DIAPAUSE IN KHAPARA BEETLE LARVAE

A.1 Method

To induce diapause, newly-hatched first instar larvae were held in Petri dishes (100 mm diameter x 15 mm height) at densities of 1, 7, 25, 110, 230, 330, and 440 larvae per 6 g diet. The lowest density was replicated 22 times, whereas all other density treatments were replicated six times. The conditions of rearing were 16:8 hour (light:dark) photoperiod at 30 ± 2 °C and 20 ± 10 % RH. Starting on Day 20 and thereafter every three days, Petri dishes were checked for the presence of pupae, adults or dead larvae. Previous studies report that non-diapausing larvae held at 30 °C will pupate after 30-35 days (Nair & Desai, 1972; Wilches *et al.*, 2017). Thus, for the purposes of this study, I identified larvae that had not pupated after 45 days as being in diapause.

A.2 Statistical analysis

To analyze the data obtained from diapause induction study, I used an approach similar to that described by (Wilches *et al.*, 2017). Individuals that survived under different densities from each treatment group was determined by the following formula:

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

A generalized linear model (GLM) with quasibinomial distribution (to account for over-dispersion in data), in place of binomial distribution, was used to determine whether there was a significant effect of larval crowding on the proportion of diapause. Initial density of the larvae was considered as an explanatory variable, proportion of diapause was the response variable. Proportion of diapause was calculated using the formula:

$$\text{Proportion of diapause} = \text{Number of diapausing larvae}/\text{Total survivors}$$

A.3 Results

The model generated from the results obtained in diapause induction study identified a percentage of diapause induction of 40% or higher when larvae were reared at densities of 38 larvae/g of diet ($F = 268.57$, df = 1, 56; $P = 0.0001$; $n = 57$; Fig. A.1). The generated model had coefficients of -2.270 (intercept), slope of 0.046 (no. of larvae/g of diet), and 4.137 as an estimated parameter of overdispersion.

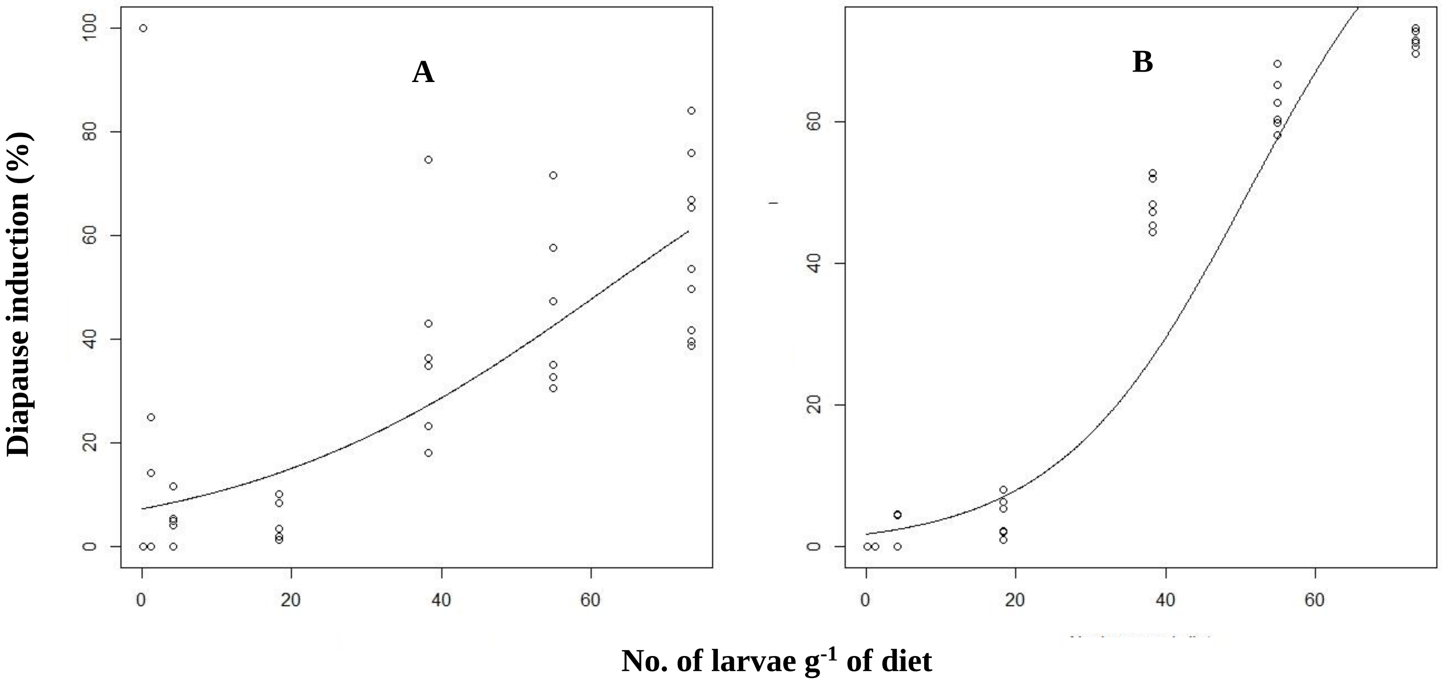

Figure A.1 Logistic regression curve (quasibinomial distribution) for percent diapause induction in khapra beetle larvae reared at different densities; **A)** Wilches *et al*. 2017, **B)** present study