Effects of Methoprene on *Tribolium castaneum* (Herbst)

(Coleoptera: Tenebrionidae)

by

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Dedicated to my

Mother,

Father (deceased),

two Sisters

and

Cousin (deceased)

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Abstract

Due to concerns with synthetic neurotoxic insecticides used in insect pest management, alternative control methods are sought. Methoprene is a juvenile hormone analogue, which is well-known for its lethal effects on immature insects, and is registered as a stored-grain insecticide in the U.S.A. and Australia. However, less is known about its sub-lethal effects. Experiments were conducted to investigate the effects of methoprene (Diacon II) on heat tolerance, cold tolerance and progeny production of *Tribolium castaneum* (Herbst) when late-instar larvae or adults were exposed to wheat treated with methoprene. Methoprene at 3.33 ppm or higher, reduced heat tolerance of adults at 46ºC. In contrast, methoprene did not affect the heat tolerance of larvae. Cold tolerance and cold acclimation of both adults and larvae was not affected by methoprene. Exposure of larvae to 0.001 or 0.0165 ppm of methoprene on wheat had 37 and 72% reduction in adult emergence, respectively. The surviving adults had normal movement, but their progeny production was reduced by 71%. Males were affected more than females. Exposure of adults to methoprene (66.6 ppm) did not reduce progeny production. Methoprene is used as a surface treatment to control insects in empty grain bins, processing facilities and warehouses. Experiments were conducted to assess the effect of surface material, temperature and cleaning practices on methoprene residual efficacy. A bioassay with late-instar larvae at 30ºC until adult emergence was used to measure methoprene residual efficacy. Methoprene applied at the label rate (0.0003 mg/cm^2) on varnished wood, held at 20, 30 or 35ºC for 24 weeks, prevented all development of larvae into adults. Conversely, concrete surfaces allowed 22% adult emergence after 8 weeks and 69% after 24 weeks. Temperatures, 20, 30 or 35ºC, that the concrete surfaces were

held before the bioassay, did not affect this decline in efficacy. Presence of flour or repeated removal of flour (cleaning) reduced the efficacy of methoprene on concrete surfaces, but not on varnished wood. Maintaining concrete surfaces at 65ºC for 48 hours did not reduce efficacy. The implications of these results are discussed in the context of controlling stored-product insects, as well as insect physiology.

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Chapter 1. Introduction

Insect pests infesting stored products were reported as early as 2500 B.C. (Sokoloff 1972). These insects are classified in the orders Coleoptera (beetles), Lepidoptera (moths) and Psocoptera (booklice). They are found in both raw and processed food, and have adapted to feed on a wide array of stored commodities: cereal grains, legumes, tobacco, dried fruits and nuts, wood and even cloth fabric or carpet (Hagstrum and Subramanyam 2006). Stored-product insects are found at different locations of the post-harvest pathway: in warehouses, elevators, flour mills, processing plants and stores (Smith and Loschiavo 1978; Doud and Phillips 2000; Arthur et al. 2006; Hagstrum and Subramanyam 2006). Many stored-product insects can crawl or fly long distances (Hagstrum and Smittle 1980; Campbell et al. 2002; Hagstrum and Subramanyam 2006), which enables them to spread to new unexploited habitats. The damage caused by stored-product insects is diverse. Feeding by insects causes weight loss. They also cause qualitative losses such as nutrient loss; reduced seed viability; contamination of food or food products by the presence of living or dead insects, insect parts, webbing or feces of insects; development of hot spots; and health risks such as pathogenic microbial infection (Hill 1990) and allergic reactions (Campbell et al. 2004). All these lead to reduced market value and economic loss (Hagstrum and Subramanyam 2006).

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is a serious insect pest of stored products (Arthur 2006), and is found in many places in bulk grains and in the distribution channel associated with processed grain products (Zettler 1991; Mullen 1992; Arbogast et al. 2000; Rees 2004; Hagstrum and

Subramanyam 2006). *Tribolium castaneum* can consume only damaged seeds but not whole seeds. However, it feeds on a wide array of raw and processed food materials: grains, oil seeds, pulses, flour, wheat bran, dried fruits, nuts, milk powder, food spices and confectionaries (Sinha and Watters 1985; Hagstrum and Subramanyam 2006). At high population densities, adults and larvae cannibalize their own eggs and pupae (Sokoloff 1974). The rate of development depends on temperature, humidity (Sokoloff 1974) and food (Hagstrum and Subramanyam 2006). At 28.5°C and 65% relative humidity (r.h.), completion of development of eggs, larvae and pupae takes 3.8, 22.8 and 6.2 days, respectively (Sokoloff 1974). Thus at 28.5°C, approximately 33 days are required for the development from egg to adult.

Current control measures for *T. castaneum* and other stored-product insects include the use of contact insecticides (Arthur and Dowdy 2003), diatomaceous earth (Dowdy 1999b), fumigation (Rajendran 2000; Reichmuth et al. 2003; Emekci 2010) and extreme temperatures (Fields 1992; Arthur 2006; Beckett et al. 2007). Limitations to the currently available control methods, especially the use of synthetic neurotoxic chemicals, include health concerns, resistant insect populations (Arthur 1996), high cost (Hagstrum and Subramanyam 2006) and negative impact on non-target organisms (Hagstrum and Subramanyam 2006). Deregistration of insecticides for stored-product insect pest management has also occurred (Snelson 1987; Fields and White 2002). Therefore, alternative and safer management methods are required. Insect growth regulators (IGRs) are compounds that imitate insect hormones. As insecticides, the IGRs limit pest populations by preventing adult emergence, and are less toxic to non-target organisms (Oberlander and Silhacek 2000).

Methoprene is an IGR that acts as a juvenile hormone analogue (JHA) (Arthur 2007; Chanbang et al. 2008); it arrests the immature stages of insects during their development to the adult stage (Oberlander and Silhacek 2000). However, JHAs do not kill adult insects (Oberlander and Silhacek 2000). There has been extensive research regarding toxicity to immature stages of methoprene (McGregor and Kramer 1975; Loschiavo 1976; Manzelli 1982; Samson et al. 1990; Nayar et al. 2002) or other JHAs (McGregor and Kramer 1975; Loschiavo 1976; Samson et al. 1990; Shaaya 1993; Nayar et al. 2002). Methoprene can kill larvae and pupae as well as stop their development without killing them (Edwards et al. 1988; Shaaya and Pisarev 1986). Thus, the majority of early research with JHAs, since their introduction in the 1970s, focussed on their lethal effects on the development of immature insects. However, sub-lethal effects of JHAs are important as they alter normal insect physiology such as diapause induction (Evenden et al. 2007) and reproduction (Masner et al. 1968; Metwally and Landa 1972; Metwally et al. 1972).

Effects of insect hormones or their analogues on tolerance of extreme temperatures are not well-studied. There are a few studies that examined the effects of hormones (JH, 20-hydroxyecdysone and adipokinetic hormone (AKH)) on the abundance of cryoprotectants in insects, when exposed to cold temperatures (Zachariassen 1980; Horwath and Duman 1983; Hamilton et al. 1986; Rojas et al. 1987; Xu et al. 1990; Xu and Duman 1991). However, these studies did not test the effect of hormones on low temperature survival. Low temperature to control stored-product insects is used in several ways including ambient air aeration of grain bins (Burks et al. 2000), grain chillers (Fields and White 2002) and freeze-out of flour mills (Burks et al. 2000). However,

limitations to the use of low temperatures include damage to equipment and energy cost (Burks et al. 2000). If JHAs reduce cold tolerance, then a combination of JHA and cold may eliminate these issues that limit the use of low temperature to control stored-product insects. The effect of JH or JHAs on insect heat tolerance has not yet been studied. Research should address the heat tolerance of both the larvae and adults, as heat tolerance differs with life stage (Fields 1992; Tang et al. 2007). With the increased use of heat treatments in flour mills to replace methyl bromide, the effect of heat on the residual efficacy of methoprene would be useful information for integrated pest management (IPM) programs in flour mills, processing plants and warehouses. Studying the effects of methoprene in combination with heat or cold treatment is justified as IGRs are better used integrated with other pest management tools than used alone (Oberlander et al. 1997). There are several examples of combining two control methods to suppress insect populations, that if used alone would not be sufficient to control population (Banks 1987; Fields 1993; Arthur 1994b; Mueller 1994; Pospischil and Smith 1994; Dowdy and Fields 2002; Hou et al. 2004). Juvenile hormone or JHAs can also reduce progeny production of certain stored-product insect species by affecting mating, fecundity, fertility or embryonic development (Oberlander et al. 1975; Marzke et al. 1977; Allanson and Wallbank 1994; Daglish and Pulvirenti 1998; Chanbang et al. 2008). However, there has been no study of such effects on *T. castaneum*.

In my dissertation research, I explored how the JHA methoprene interferes with selected physiological processes in *T. castaneum*, and how these findings can be used to understand JH regulation of physiological processes and to control this widespread

stored-product insect pest. The following questions were examined using *T. castaneum* as the test insect and methoprene as the JHA.

1. Does methoprene affect susceptibility to high temperatures?
2. Does methoprene affect susceptibility to low temperatures?
3. Does methoprene affect progeny production?
4. What are the effects of material, temperature of exposure, duration of exposure and sanitation on the residual efficacy of methoprene?

In this dissertation, the research conducted to examine these questions is included as separate chapters, following Chapter 2, which reviews the literature. Research on questions 1 and 2 is included as Chapter 3. This is modified from: Wijayaratne, L.K.W., Fields, P.G., 2010. Effect of methoprene on the heat tolerance and cold tolerance of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Journal of Stored Products Research 46: 166-173 (doi: 10.1016/j.jspr.2010.04.001). Chapter 4 includes the research that examined question 3. This is modified from Wijayaratne, L.K.W., Fields, P.G., Arthur, F.H., 2011. Effect of methoprene on the progeny production of *Tribolium castaneum* (Coleoptera: Tenebrionidae). Pest Management Science (in press, doi: 10.1002/ps.2247). Chapter 5 includes the research related to question 4, and I intend to submit this to Journal of Economic Entomology.

At the initial stages, my Ph.D. dissertation was intended to examine the effects of methoprene on both *T. castaneum* and *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae). These two species were selected because they represent two widely different taxonomic groups of stored-product insects. *Plodia interpunctella* has a larval diapause, *T. castaneum* does not. Therefore, one of the early objectives was to determine if

methoprene affects diapause induction in *P. interpunctella*. As the first step for this study, experiments were launched to obtain a population that exhibits diapause, by using four *P. interpunctella* populations that originated from different geographical regions. As there was a low frequency of diapause induction, even at low temperatures and short day length, experiments were set up to select for a population with high diapause. Although frequency of diapause was increased by selection, the experiment had already consumed two and a half years of the study program. Therefore, the effect of methoprene on diapause induction in *P. interpunctella* was not studied due to time restrictions. However, the findings on diapause induction in *P. interpunctella* are included as an appendix to the dissertation.

Scientific contribution

Wolly Wijayaratne developed the research questions and protocols for experiments, designed and carried out the experiments, analyzed the data, and wrote the preliminary drafts of all the papers that were or will be submitted to scientific journals. The co-authors, Paul Fields and Frank Arthur, reviewed and modified experimental designs and edited the manuscripts before submission. The other Ph.D. committee members Drs. Neil Holliday, Desiree Vanderwel and Steve Whyard provided general comments on experimental designs.

Chapter 2. Literature Review

Juvenile hormones

The development of insects is controlled by two hormones: JH and ecdysteroids (Nation 2002; Keshan et al. 2006). Juvenile hormone is a sesquiterpenoid (Erezyilmaz et al. 2004), which is secreted by the corpora allata (Granger et al. 1979), a pair of endocrine glands located just posterior to the brain. Ecdysteroids are secreted from the prothoracic gland (Nation 2002). Juvenile hormone regulates a wide array of physiological processes. Its well-known function is to prevent metamorphosis (Konopova and Jindra 2007; Minakuchi et al. 2008a; Minakuchi et al. 2009; Parthasarathy and Palli 2009; Zhou and Riddiford 2002). During larval molting, there is a critical period during which JH must be present (Truman 1972; Fain and Riddiford 1976). The relative differences in the concentrations of JH and ecdysteroids determine the type of molting during insect development (Nation 2002). In early instars of hemimetabolous insects, JH is secreted prior to the increase in ecdysteroid concentration, and the JH concentration decreases towards the end of the instars. This mediates the secretion of a new nymphal cuticle. In the last instar, JH concentration decreases to a minimum level. Higher concentration of ecdysteroids causes last-instar nymph to molt into an adult (Nation 2002). In holometabolous insects, JH concentration is high compared to ecdysteroid in the early stage of an instar. Later, immediately prior to molting, JH concentration decreases but is still maintained at moderate levels. This causes a larval-larval molt (Nation 2002). Methyl farnesoate is the precursor of JH (Teal and Proveaux 2006). Its production is low in the early last-larval instar of holometabolous insects (Bhaskaran et al. 1986), and consequently the JH concentration is also reduced during this period

(Nation 2002). Increased ecdysteroid concentration at this time induces metamorphosis in these insects (Nation 2002). The effects of JH differ with insect taxa (Zhou and Riddiford 2002). In Coleoptera and Lepidoptera, JH averts the changes during metamorphosis (Riddiford 1995; Zhou and Riddiford 2002) and thus confers the 'status quo' (Zhou and Riddiford 2002). Conversely in Diptera, JH does not prevent the metamorphic transitions between larva and pupa (Riddiford and Ashburner 1991). However, in these dipterans, the presence of JH in the final larva or the pre-pupa disturbs both the metamorphosis in some systems (Restifo and Wilson 1998) and the normal differentiation of certain adult characters (Riddiford and Ashburner 1991).

Beside the aversion of metamorphosis, JH also regulates many different functions across a wide range of insect taxa (Sieber and Benz 1980; Parthasarathy et al. 2010b; Bhaskaran 1972; Wigglesworth 1969; Lüscher 1972; Nemec et al. 1970; Beckage and Riddiford 1982; Chamberlain and Hopkins 1970). Some of the most common functions are effects on reproduction in males (Parthasarathy et al. 2009) and females (Parthasarathy et al. 2010a), vitellogenin synthesis (Koeppe et al. 1985), embryonic development (Ishaaya and Horowitz 1992), diapause (Yagi and Fukaya 1974), communication (Kim et al. 2005), polymorphism (Schmidt Capella and Hartfelder 1998), and behaviour during feeding (Sieber and Benz 1978) and mating (Oberlander et al. 1975). Depending upon the species, JH can have different effects on the same physiological process. For instance, JH induces diapause in larvae of *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae) (Eizaguirre et al. 1998) and *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) (Yagi and Fukaya 1974). Conversely, JH terminates diapause in adults of *Leptinotarsa decemlineata* Say (Coleoptera:

Chrysomelidae) (Schooneveld et al. 1977) and *Draeculacephala crassicornis* Van Duzee (Homoptera: Cicadellidae) (Kamm and Swenson 1972). Juvenile hormone regulates the synthesis and uptake of vitellogenin in *Pseudaletia unipuncta* (Haworth) (Lepidoptera: Noctuidae) (Cusson et al. 1994), *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Satyanarayana et al. 1992) and *Heliothis virescens* F. (Lepidoptera: Noctuidae) (Zeng et al. 1997). In contrast, JH does not regulate vitellogenesis in *Bombyx mori* (L.) (Lepidoptera: Bombycidae) (Tsuchida et al. 1987).

Juvenile hormone or its precursor methyl farnesoate also affects other organisms. These include crustaceans such as barnacles, shrimps and crabs (Olmstead and LeBlanc 2003); annelids (Laufer and Biggers 2001); and nematodes (Rogers 1979). The effects of these compounds on organisms other than insects include increased fecundity in shrimp (Olmstead and LeBlanc 2003), metamorphosis promoting effects in annelids (Laufer and Biggers 2001) and inhibition of egg hatching in nematodes (Rogers 1979).

Forms of juvenile hormones

There are four major forms of the juvenile hormone: JH 0, JH I, JH II and JH III (Schooley and Baker 1985). The differentiation is based on the identity of the aliphatic group on the main chain. In Lepidoptera JH 0, I and II are present, whereas JH III is reported mostly in other insects (Schooley et al. 1984). JH III bisepoxide is found in higher Diptera (Richard et al. 1989), and methyl farnesoate is found in the eggs and larvae of cockroaches (Bruning et al. 1985). In the cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera**:** Noctuidae)**,** JH II is the dominant form of JH (Fan et al.

1999). Beside these different forms, there are also geometric isomers of JH: JH I has trans-2, trans-6 and cis-10 forms (Staal 1975).

Juvenile hormone and its metabolism

Juvenile hormone synthesis (Audsley et al. 1999; Li et al. 2002; Mayoral et al. 2009) and degradation (Hammock 1985) are regulated by the corpora allata, in response to internal and external signals. These signals are integrated in the neurosecretory cells of brain (Feyereisen 1985). During insect development, JH is first produced in the embryonic stage after dorsal closure (Bürgin and Lanzrein 1988). The synthesis of JH is affected by both external and internal factors such as photoperiod, nutrition, mated status, regulation from the ovary, stimulatory (allatotropins) or inhibitory (allatostatins) secretions from the brain (Audsley et al. 1999; Li et al. 2002) and ecdysteroid concentration (Steel and Davey 1985; Richard and Gilbert 1991). Following its synthesis, JH is not stored in corpora allata but is released into hemolymph (Feyereisen 1985).

Once JH is released from the corpora allata, JH binds to juvenile hormone binding proteins (JHBPs) or to hemolymph protein (Zalewska et al. 2009). The JHBPs are either low-molecular-weight-binding proteins (LMBPs) or high-molecular-weight-binding proteins (HMBPs) (Goodman and Chang 1985; Duk et al. 1996). These JHBPs compete for different forms of JH and bind them with different affinities in different species (Tawfik et al. 2006). Certain HMBPs bind JH III with higher affinity (Bertuso and Tojo 2002) than JH I (Ismail and Gillott 1996) and JH II (Trowell et al. 1994). In contrast, some other JHBPs bind JH I and JH II with high affinity and JH III weakly (Goodman and Chang 1985; Goodman 1990). The JHBPs prevent enzymatic degradation (Goodman

1990). In addition, JH may also be bound to other hemolymph proteins such as arylphorin (Goodman and Chang 1985; Goodman 1990). The JHBPs and hemolymph proteins transport JH to the target tissues to cause biological effects (Zalewska et al. 2009; Gilbert et al. 2000). Epidermis (Muramatsu et al. 2008), ovary (Bertuso and Tojo 2002), male accessory glands (Parthasarathy et al. 2009), fat body (Paes-de-Oliveira et al. 2008), muscles (Kobayashi and Ishikawa 1994), nervous system (Truman and Reiss 1988) are the main targets for JH. Degradation of JH is carried out mainly by specific juvenile hormone esterases (JHE) (Tsubota et al. 2010) and JH epoxide hydrolase (Mackert et al. 2010).

Once JH reaches the cell, it operates in various ways. Molecules of JH act at the membrane level (Van Mellaert et al. 1985; Van Mellaert et al. 1989), and are likely to induce the phosphatidylinositol pathway. Intracellular binders for JH are found in fat body, epidermis, ovary, accessory glands and intracellular binding of JH is evident at these locations (Goodman and Chang 1985; Goodman 1990; Palli et al. 1991; Riddiford 1994). In *Drosophila* cell lines, the JH molecule binds to cytosolic JH binding proteins (Wang et al. 1989; Shemshedini et al. 1990) and nuclear receptors (Braun et al. 1995) inside the cell. Actions of JH take place both at cellular and molecular levels. A wide array of research has been conducted to discover the molecular basis of JH in its different functions such as binding with JHBP (Sok et al. 2008), prevention of metamorphosis (Konopova and Jindra 2007; Parthasarathy and Palli 2009), vitellogenin synthesis (Parthasarathy et al. 2010b) and degradation of JH (Mackert et al. 2010; Tsubota et al. 2010).

There is evidence that JH modifies the effects of ecdysone (Maki et al. 2004). Juvenile hormone affects the level of expression of ecdysone receptor (EcR) and ultraspiracle (Usp), two genes regulated by ecdysteroid (OżYhar et al. 1991). Also, both JH and ecdysteroid affect the E75 gene (Sok et al. 2008). The expression of E75A protein is induced by JH, and E75A can modify the metamorphic actions driven by ecdysteroid, when JH is present (Dubrovsky et al. 2004). The gene BR-C is induced by 20-hydroxyecdysone and this induces a larva to larva molt in the presence of JH (Sok et al. 2008) by expression of the protein, E75A (Dubrovsky et al. 2004). During the last larval instars in holometabolous insects, a small peak in the 20-hydroxyecdysone regulates activities leading to pupal commitment such as cessation of feeding and inception of pre-metamorphic behavior (Nijhout 1994). Simultaneously, a number of transcription factors are up regulated (Russell and Ashburner 1996; Thummel 1996). Up regulation of the transcription factor (and the gene) Br during the final larval stage is necessary for the larval-pupal transformation (Parthasarathy et al. 2008). Activation of BR-C in the absence of JH causes pupal commitment (Sok et al. 2008). Expression of Br by JH has been shown in the epidermis of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) and *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) during pupal-adult molt (Zhou and Riddiford 2001).

Insecticides based on insect hormones

Insecticide development has undergone several eras. Early insecticides were based on elements such as sulfur and lead or compounds based on these elements, such as lead arsenate. Subsequent to that, organochlorines, organophosphates, carbamates and

synthetic pyrethroids were synthesized (Williams 1967; Ware and Whitacre 2004). This was followed by the synthesis of insecticides based on plant-derived compounds (Ware and Whitacre 2004) and insecticides based on insect hormones (Williams 1967).

The term "insect growth regulators" (IGRs) was defined by Carroll Williams to describe insecticides based on insect JH (Williams 1967). Later, some other compounds mimicking ecdysteroids (Wing 1988), and those affecting chitin synthesis (Post and Vincent 1973) were also synthesized. Thus, in the broad sense, IGRs interfere with three different types of physiological processes: developmental regulation, moulting initiation and chitin synthesis (Oberlander and Silhacek 2000). Insect growth regulators that hinder these three different processes are designated as juvenile hormone agonists, ecdysteroid agonists and chitin synthesis inhibitors, respectively (Oberlander et al. 1997). The IGRs usually have no adverse effects on humans or the environment, are compatible with other pest management strategies, and also have different modes of action from most insecticides (Staal 1975).

Juvenile hormone analogues

Compounds and chemical structures

Juvenile hormone analogues is the first group of IGRs discovered and used in pest management (Oberlander et al. 1997). For research purposes, JHAs are good substitutes for JH, as these compounds withstand degradation that natural JH undergoes in insects (Henrick et al. 1973). Several products of JHAs have been commercially developed. They are methoprene by Zoecon, Novartis and Wellmark in 1973 (Oberlander and Silhacek 2000); hydroprene by Zoecon in 1973 (Henrick et al. 1973); fenoxycarb by Magg and

Novartis in 1981; and pyriproxyfen by Sumitomo in 1984 (Oberlander and Silhacek 2000). Methoprene was the first JHA to be synthesized and tested against insect pests. Furthermore, it is the JHA closest in structure to natural JH. Both methoprene and hydroprene are aliphatic compounds, and have some degree of structural similarity. They share properties such as chemical stability and resistance to degradation within the insect body (Henrick et al. 1973). In contrast, fenoxycarb and pyriproxyfen are aromatic compounds (Riddiford 1994). Structures of JHAs have been determined: methoprene is isopropyl 11- methoxy (2*E*,4*E*), 3,7,11 trimethyl-2-,4-dodecadienoate (Wilson and Turner 1992); hydroprene is ethyl (*2E-4E*)-3,7,11 trimethyl-2,4-dodecadienoate (Gupta and Mkhize 1982); fenoxycarb is (ethyl (2-(phenoxy-phenoxy) ethyl) carbamate (Thind and Edwards 1986); and pyriproxyfen is 4-phenoxyphenyl (R,S)-2-(2-pyridyloxy) propyl ether (Hatakoshi 1992) or 2-(1-methyl-2-(4-phenoxyphenoxy) ethoxy) pyridine (Ishaaya et al. 1994). The JHAs are believed to share modes of action similar to that of JH and to have similar cellular/nuclear receptors as for JH, although these have not yet been fully elucidated (Riddiford 2008). Also, JHAs have low vertebrate toxicity (Wright 1976).

Biological effects of juvenile hormone analogues

When JH or JHAs are externally applied to insects, the normal regulation of JH concentration in the insect body is affected, leading to a variety of morphological and physiological alterations compared to a healthy insect (Loschiavo 1976; Nijhout and Wheeler 1982; Ignell et al. 2001; Kim et al. 2005; Bai et al. 2010; Parthasarathy et al. 2010b; Athanassiou et al. 2011). Such biological effects caused by an externally-applied JHA depends on several factors: species-specific activity of the type of JHA, insect life-

stage sensitivity, rate of penetration through cuticle, binding of the molecule to the carrier proteins in hemolymph, ability to withstand degradation enzymes, compatibility of JHA molecule with the receptor and the action on endocrine and other systems (Staal 1975).

The most common effects of JHAs are production of supernumerary instars (Retnakaran 1973; Shaaya and Pisarev 1986), larval-pupal intermediates (Hong 1975), pupal-adult intermediates (Loschiavo 1976; Hong 1975), and delay in the larval-pupal transformation (Shaaya and Pisarev 1986). The JH inducible transcription factor *Kruppel homolog 1* (*Kr-h1*) expression is induced by JHAs during the pupal-adult transition of *D. melanogaster* (Minakuchi et al. 2008b). This *Kr-h1* is responsible for the prevention of metamorphosis during larval and pupal stages (Minakuchi et al. 2009).

Juvenile hormone analogues have been screened against many stored-product insect pests in Coleoptera (Thomas and Bhatnagar-Thomas 1968; Strong and Diekman 1973; McGregor and Kramer 1976; Mian and Mulla 1982a; Allanson and Wallbank 1994; Bengston and Strange 1994) and Lepidoptera (Strong and Diekman 1973; Silhacek and Oberlander 1975; Nickle 1979; Shaaya 1993; Bengston and Strange 1994), and a wide array of physiological processes are affected. Generally, JHAs do not have lethal effects on adults (Oberlander and Silhacek 2000).

Resistance to juvenile hormone analogues

When the insecticides based on insect hormones were first introduced, one of the expected advantages was that the insects would not be able to develop resistance to these compounds (Williams 1967). This idea, however, was disputed from the beginning (Ellis 1968; Schneiderman 1972). Shortly afterwards, insect populations that were resistant to

other insecticides were found to be also resistant to JH or its analogues (Dyte 1972). Resistance to methoprene (Hammock et al. 1977; Benezet and Helms 1994; Ashok et al. 1998; Cornel et al. 2002) and pyriproxyfen (Bull and Meola 1994; Ishaaya and Horowitz 1995) has now been detected. *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) and (Benezet et al. 1993) and *Ochlerotatus nigromaculis* (Ludlow) (Diptera: Culicidae) (Cornel et al. 2002) exhibit resistance to methoprene.

Reduced binding of JH III to the cytosolic binding protein is shown in *D. melanogaster* (Shemshedini and Wilson 1990). Most common mechanisms of resistance to an insecticide include reduced penetration, sequestration in tissues, excretion from the body, detoxification or insensitivity of target tissue (Feyereisen 1995; Ashok et al. 1998). A gene that confers resistance to JH or JHAs has been identified and named *Methoprene-tolerant* or *Met* (Wilson and Turner 1992; Ashok et al. 1998). Binding of JH III by a cytosolic protein from the target site is lower in the *D. melanogaster* population resistant to JH III than in the susceptible flies (Shemshedini et al. 1990). This suggests the target-site resistance.

There were some early suggestions explaining the development of resistance to JHAs. These included delayed penetration of topically applied JH I across the cuticle and from there to the tissue (Edwards and Rowlands 1978), prevention of carryover effect of methoprene to the subsequent instars due to the shedding of cuticle during molting (Staal 1975) and use of same classes of enzymes for the degradation of both JH and insecticides (Staal 1975). Hence, insects that are resistant to insecticides through increased levels of mixed-function oxidases are expected also to show resistance to JHAs (Hammock et al.

1977; Hammock 1981). However, the mechanism by which an insect becomes resistant to methoprene has not yet completely elucidated (Palli 2009).

Commercial use

Insect growth regulators have been developed into commercial products and are used in agriculture, forestry and medical entomology (Mondal and Praween 2000). The JHAs are available as powder (Richardson and Lagos 2007) or emulsifiable concentrates (Chanbang et al. 2008). Methoprene was registered as an insecticide, Diacon®, in the U.S.A. in the 1980s, and contained both the R- and S-isomers (Arthur 2004). It was reintroduced by Wellmark International as Diacon® II in 2002, with only the biologically active S-isomer of methoprene (Chanbang et al. 2008). In the U.S.A., methoprene is used in different stored-product treatments: for direct application on grains; as a contact insecticide; and as an aerosol-space application in facilities such as mills, warehouses and indoor-food-storage facilities. The label rate of methoprene for surface applications is 0.0003 mg/cm^2 (Jenson et al. 2009). In Australia, methoprene is registered as a grain protectant (Daglish and Nayak 2010). Pointsource™ (Arthur 2003), Gentrol® (Arthur 2001) and Protrol® (Bell and Edwards 1999) are commercial products of hydroprene in the U.S.A. and U.K. Recently, pyriproxyfen was also labelled in the U.S.A. as a contact insecticide on floors and is available as NyGuard® (Arthur and Phillips 2009). Methoprene was first registered in Canada in 1977 for mosquito control (Health Canada 2001). It is not yet registered in Canada for stored-product use.

Cold tolerance

Effects of temperature on stored-product insects

In insects, almost all activities are influenced by temperature. In the broad sense, these include cellular enzymatic reactions, various physiological processes, behaviour and evolution (Lee 1991). For stored-product insects, temperatures of 25-33ºC are optimum for normal development and reproduction (Fields 1992). Most of the insects from this group can complete their development at temperatures as low as 13ºC or as high as 35ºC, although the rate of development is slower than in the optimum temperature range. Temperatures below 13ºC and above 35ºC are not favorable for development of these insects, and they will eventually die (Fields 1992). Longer durations and more extreme temperatures cause greater or faster mortality (Fields 1992).

Reasons for insect mortality at low temperatures

There are several explanations for the lethality at low temperatures. When the temperature drops, viscosity of phospholipid membranes of cells decreases (Hazel 1989; Storey and Storey 1989). At low temperatures, some saturated lipids in the phospholipid membranes form clusters (Wunderlich et al. 1978). Weak-bond interactions among proteins are affected by low temperature and cause conformational changes or denaturation of proteins (Storey and Storey 1989). Phospholipid membranes are the outer limits of cells, are a component of cellular organelles, and membrane-bound enzymes are embedded within them. Salt concentrations increase and decrease during freezing and thawing, respectively, resulting in an osmotic shock (Storey and Storey 1989).

Furthermore, freezing adversely affects the movement of molecules inside and between the cells (Storey and Storey 1989).

Insect responses to low temperatures

When an insect's body temperature decreases, formation of ice crystals, or freezing, takes place at sub-zero temperatures (Lee 1991; Denlinger and Lee 1998; Lee 2010). Based on the survival at the freezing temperatures, insects are categorized as freeze-intolerant and freeze-tolerant insects. Freeze-intolerant insects die with the formation of ice within their bodies (Lee 1991). In contrast, freeze-tolerant insects can withstand extra-cellular freezing in the body (Storey and Storey 1988; Fields 1992). Supercooling is the maintenance of body fluid in the liquid state, below the freezing point of the solution. Supercooling point (SCP) is the temperature at which freezing occurs in an insect that is supercooled. In freeze-intolerant species, the SCP is the lower lethal temperature (Lee 2010). Conversely, in freeze-tolerant species, the SCP represents a temperature at which their normal physiological processes start to change (Lee 2010). Supercooling capacity is the temperature difference between the SCP and the body fluid melting point. During exposure to freezing temperatures, freeze-intolerant insects increase their supercooling capacity by lowering their SCP, as an adaptation to withstand freezing (Lee 2010). In contrast, freeze-tolerant insects lower their supercooling capacity by increasing the SCP (Lee 2010). This protects them from intracellular freezing as the ice nucleation takes place outside the cell at warm sub-zero temperatures (Lee 1991). This sort of regulation of SCP is achieved by various physiological and biochemical mechanisms (Lee 1991). Supercooling point differs with the species (Lee 1991). Within a

species, the SCP differs with life stage (Carrillo and Cannon 2005) and cold acclimation (Carrillo et al. 2005). However, it is important to understand that the SCP alone is not a good representation of insect cold tolerance (Fields 1992; Carriilo et al. 2005). Thus, certain species with low SCP do not survive freezing, whereas some other species with high SCP survive (Lee 1991; Turnock and Fields 2005).

The well-known physiological responses of insects to low non-freezing temperatures are slower movement (Lee 1991), alterations in development, failure in reproduction and death (Denlinger and Yokum 1998). Exposure to low temperature causes developmental and reproductive alterations, such as production of multiple embryos (Miya and Kobayashi 1974), increase (Cymborowski and Bogus 1976) or decrease (Denlinger and Lee 1998) in the number of larvae produced, reduced size (weight) of larvae or pupae (Baker 1983), absence of release of sperm from the spermathecae (Denlinger and Lee 1998) or reduced fecundity (Hutchinson and Bale 1994). Cold tolerance, also known as cold hardiness, is the ability of an organism to survive at low temperatures. Insect cold hardiness is determined by a number of factors: temperature of exposure, period of exposure, temperature of previous exposure, stage of development, nutritional status and genetics of the insect (Lee 1991). Cold acclimation is the increase in cold hardiness due to the exposure of insects to non-lethal cool temperatures (Smith 1970; Fields 1990; Fields 1992).

Physiological and biochemical mechanisms of insect cold tolerance

During cold exposure, physiological and biochemical processes in insects, both freeze tolerant and freeze intolerant, operate concurrently to increase cold tolerance. The major mechanisms for cold tolerance are accumulation of low-molecular-weight cryoprotectants such as glycerol (Ishiguro et al. 2007), sorbitol (Pullin et al. 1991; Morason et al. 1994), trehalose (Zeng et al. 2008), galactose (Zeng et al. 2008), glucose (Zeng et al. 2008), erythritol (Baust and Edwards 1979), ethylene glycol (Gehrken 1984); synthesis of anti-freeze proteins (thermal-hysteresis proteins) (Tyshenko et al. 1997; Kristiansen et al. 2005); and production of ice-nucleating agents (Castrillo et al. 2000). However, there are differences in the mechanisms of cold tolerance between freeze-tolerant and freeze-intolerant species (Lee 1991).

Adaptations in freeze-intolerant insects include elimination of gut contents (Boiteau and Coleman 1996); removal (Baust and Zachariassen 1983) or inactivation (Lee 1991) of ice nucleators in cells; or removal of incidental nucleators in the body to prevent freezing (Lee 1991). Furthermore, freeze-intolerant insects may synthesize anti-freeze proteins (thermal-hysteresis proteins). These depress the melting point of body fluid (Tyshenko et al. 1997); reduce hemolymph freezing point and SCP (Duman 1982); or prevent recrystallization of ice crystals (Knight and Duman 1986) and devitrification during thawing (Lee 1991). Recrystallization is the redistribution of ice crystals and devitrification is the 'process of nucleation from a glassy state' (Duman et al. 1991b, p. 122). Thermal-hysteresis proteins inhibit the growth of ice crystals by adsorption to the embryonic ice crystals (Tyshenko et al. 1997). Freeze-intolerant insects also synthesize polyols (Ishiguro et al. 2007) and sugars (Shimada et al. 1984) that depress the melting

point and the SCP of their hemolymph. Mode of action of these polyols and sugars is to mask or inactivate endogenous ice nucleators present in insects (Lee 1991), or to reduce freezing point though their colligative properties (Fields 1992).

The main adaptations that induce cold tolerance in freeze-tolerant insects include presence of ice nucleators such as hemolymph lipoproteins (Duman et al. 1991a) and ice-nucleating proteins (Wilson and Ramlov 1995) and accumulation of low-molecular-weight cryoprotectants such as polyols and sugars (Zeng et al. 2008). Hemolymph ice nucleators prevent intracellular ice formation by triggering ice formation in the hemolymph (extra-cellularly) at high subzero temperatures, usually above −10°C. This reduces the damage from osmotic shock by facilitating the slow removal of water from cells (Zachariassen and Hammel 1976; Duman and Horwath 1983; Lee 1991). Extra-cellular ice formation induces the osmotic dehydration inside cells, and avoids cell contents being frozen (Sincliar and Renault 2010). Also, the extra-cellular ice formation lowers freezing point (Gehrken and Southon 1992), and avoids the supercooling of the cell and the hazards associated with that supercooling (Lee 1991). Furthermore, the cell membrane prevents propagation of extra-cellular ice into the intracellular environment and so prevents intracellular freezing (Zachariassen and Hammel 1976). Even though the ice formation happens initially extra-cellularly, the continued supercooled status inside the cell may lead to intracellular ice formation (Duman et al. 1991b).

In certain species cold tolerance is linked with diapause. For instance, adult *Drosophila montana* Stone, Griffen and Patter (Diptera: Drosophilidae) entering reproductive diapause has higher cold tolerance than non-diapausing adults (Vesala and Hoikkala 2011). Diapausing *P. interpunctella* larvae are more cold tolerant than non-

diapausing larvae (Naeemullah et al. 1999). In *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae), cold tolerance is higher in diapausing pupae than in non-diapausing pupae (Lee and Denlinger 1985). The physiological changes associated with the diapause make these insects more resistant to low temperatures (Lee 1991; Colinet et al. 2010). Synthesis of stress proteins in response to cold exposure is observed in *S. crassipalpis* (Joplin et al. 1990), *D. melanogaster* (Burton et al. 1988) and *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) (Denlinger et al. 1992). More recent literature reveals the up regulation of heat shock proteins (HSPs) under cold stress. In *D. melanogaster*, the expression of HSP 70 is increased after cold exposure, but that of HSP 23 remains unchanged (Sinclair et al. 2007).

Influence of the environment on the biochemistry within the insect body is explained by Hochachka and Somero (1984); Storey and Storey (1989); and Fields (1992). The rates of biochemical reactions are reduced at lower temperatures, compared to those at optimum temperatures. As biochemical reactions are connected, and the rate of change due to temperature may be different between reactions, these reactions can become uncoupled. Consequently, imbalances in the biochemical reactions resulting from such rate changes during cold exposure can cause injury. A second mechanism involves changes in the ionic activity at low temperatures. For example, the hydrogen ion concentration and the solubility of oxygen in water increase at lower temperatures, and both these changes will affect enzymatic activity in insects (Fields 1992). So, species that exhibit increased cold tolerance must tolerate these biochemical changes.

The most prominent biochemical reactions that deal with cold tolerance include enzymes such as phosphotases, phosphorylases and phosphofructokinases (Storey and

Storey 1991). When temperature increases, cryoprotectants are broken down; polyols are reconverted to glycogen, converted to trehalose or incorporated in biosynthesis of lipids and amino acids (Lee 1991). Catabolism of cryoprotectants is mediated by enzymes such as dehydrogenases (Lee 1991).

Hormonal regulation of cold tolerance

There have been few studies exploring the hormonal control of insect cold tolerance (Kidokoro et al. 2006; Cho et al. 2007), and these studies tested only the effect of hormones on cryoprotectants (Horwath and Duman 1983; Hamilton et al. 1986; Xu et al. 1990; Xu and Duman 1991; Watanabe and Tanaka 1999; Cho et al. 2007). The corpora allata, prothoracic gland and corpora cardiaca can affect the concentrations of cryoprotectants, through their hormonal products JH, ecdysteroids and AKH, respectively (Zachariassen and Lundheim 1992). The effects of these hormones on cryoprotectants vary with insect species. However, none of these studies have examined the effects of hormones on survival at low temperatures.

Of the different forms of JH, only JH I has been shown to regulate the concentration of cryoprotectants (Zachariassen and Lundheim 1992); the effect on the concentration of cryoprotectants differs with species. Treatment of larvae of *Dendroides canadensis* Latreille (Coleoptera: Pyrochoridae) with JH I increases antifreeze protein levels but decreases polyol levels in the hemolymph (Horwath and Duman 1983). In contrast, application of JH I increases glycerol levels in larvae of *C. suppressalis* (Tsumuki and Kanehisa 1980). In *Eurosta solidaginis* (Fitch) (Diptera: Tephritidae) larvae, prevention of JH reaching the abdomen by head ligation reduces accumulation of

glycerol (Hamilton et al. 1986; Zachariassen and Lundheim 1992) and increases SCP in summer-collected larvae but no such differences are seen winter-collected larvae (Rojas et al. 1987). Fenoxycarb applied to *Scotinophora lurida* (Burmeister) (Hemiptera: Pentatomidae) adults reduces glycerol content (Cho et al. 2007). Changes in the abundance of cryoprotectants in these species do not always clearly explain how JH may affect cold tolerance.

A few studies looked at the effects of ecdysone and AKH on cryoprotectants. The 20-hydroxyecdysone (20-HE) increases the SCP and glycerol level in the diapausing pupae of *S. crassipalpis* (Lee et al. 1988). In contrast, it reduces the glycerol levels in *C. suppressalis* (Tsumuki and Kanehisa 1981). Adipokinetic hormone induces release of lipoprotein ice nucleators from fat bodies to the hemolymph and so increases their ice nucleation in *Ceruchus piceus* (Weber) (Coleoptera: Lucanidae) (Xu et al. 1990; Zachariassen and Lundheim 1992). However, as *C. piceus* is a freeze-intolerant species, this increased concentration of ice nucleators is expected to reduce their survival, although this was not tested. Therefore, in general, the effects of JH, ecdysone and AKH on cold tolerance remain unclear.

Cold tolerance of stored-product insects

At 20°C, many stored-product insects cease developing (Fields 1992). They fail to reproduce at temperatures below 18°C; an exception is *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) which can reproduce at temperatures down to 15°C. Temperatures from 5 to 13°C are occasionally lethal and from 3 to 5°C, stored-product

insects stop moving. Exposure at temperatures from −5 to −10°C causes death in weeks and from −15 to −25°C causes death in minutes (Fields 1992).

In stored products, the most cold-susceptible species are *T. castaneum*, *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) and *Oryzaephilus mercator* (Fauvel) (Coleoptera: Silvanidae) (Fields 1992). *Tribolium confusum* is more cold tolerant than *T. castaneum* (Takashima et al. 1969). The moderately-cold-tolerant species are *Cryptolestes pusillus* (Schönherr) (Coleoptera: Cucujidae), *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) and *Stegobium paniceum* (L.) (Coleoptera: Anobiidae). The most cold-tolerant species are *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae), *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae), *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae), *P. interpunctella*, *S. granarius* and *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae) (Fields 1992; Fields and White 2002).

Generally, eggs are more cold susceptible than other stages. Cold tolerance also varies with the age of the eggs (Johnson and Wofford 1991). In *R. dominica* and *S. oryzae*, larvae are the most cold-tolerant stage (David et al. 1977). In *C. ferrugineus* (Smith 1970) and *T. confusum* (Nagel and Shepard 1934), the adult is the most cold-tolerant stage. In *S. granarius*, larval stages show similar cold tolerance to that of adults (Howe and Hole 1968). In *T. castaneum*, cold tolerance from most to least tolerant is adult>old larva>pupa>egg>young larva (Takashima et al. 1969). Cold tolerance is also affected by diapause status. Over 40 species of stored-product insects have a diapausing

stage (Bell 1994), and those in the family Pyralidae increase their cold tolerance during diapause (Cox 1987; Naeemullah et al. 1999; Fields and Timlick 2010). However, little research has been done on the influence of hormones on diapause induction in stored-product insects. Cold tolerance is also increased by cold acclimation. Exposure at an intermediate temperature between 0 to 20°C, before the exposure at a target low temperature, increases cold tolerance of stored-product insects by 2 to 10 times (Fields 1992; Fields et al. 1998).

Heat tolerance

Effect of high temperature on insects

Insect responses to high temperature are diverse. Exposure to sub-lethal temperatures causes 'thermal wounding' which is expressed as incomplete development, delay or failure in eclosion, or reproductive failure. Incomplete development results in deformities such as adults with abnormal shapes, colours or wing shapes, and males having feminized morphological features (Denlinger and Yokum 1998). Increased temperature affects the nervous system (Neven 2000). In *Periplaneta americana* L. (Orthoptera: Blattidae), electrical activity of nerves is increased above 30ºC, and thus the frequency of firing in the nerves is increased (Kerkut and Taylor 1957). Altered functions in the nervous system may affect the endocrine system (Neven 2000). Temperatures higher than optimum (approximately 37.5ºC) reduce both the rate of larval development and fecundity of resulting adults in codling moths *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Proverbs and Newton 1962). Furthermore, holding fifth instar larvae of *C. pomonella* at 38ºC for 4 days causes supernumerary molts (White 1981).

Mechanisms of mortality at high temperatures

Several physiological and biochemical mechanisms have been proposed to explain the lethal effects caused by increased temperatures. Increased temperatures cause abnormalities at the cellular level (Denlinger and Yokum 1998). Heat stress causes quantitative and qualitative changes in cellular macromolecules including changes in RNA (Malcolm 1969), DNA (Warters and Brizgys 1987), carbohydrates (Attifield 1987), lipids (Downer and Kallapur 1981) and proteins (Denlinger and Yokum 1998). Some of these changes have not been demonstrated specifically in insects and the generalizations are drawn based on responses in other organisms. At high temperatures, lesions are seen in DNA (Warters and Brizgys 1987). Heat-induced differences in the kinetic properties of macro molecules cause conformational changes and affect their functions. These adverse effects are sometimes irreversible (Hochachka and Somero 1984; Denlinger and Yokum 1998).

There are many reasons underlying the death of insects. Increased temperature either changes the binding of a substrate to an enzyme (Hochachka and Somero 1984) or rate of enzyme activity by altering the percentage of molecules having required energy for reactions (Hoffman 1984). Increased temperature also affects the fluidity of phospholipids bilayer in membrane-bound enzymes (Hochachka and Somero 1984). Thus, rate of an enzymatic reaction differs with the change in temperature (Hoffmann and Marstatt 1977), and interactions between enzymes differ at temperatures above 40ºC (Neven 2000). Heat disturbs ionic activities of the cells and also causes desiccation (Hochachka and Somero 1984; Fields 1992; Denlinger and Yokum 1998).

The hemolymph pH in insects is fairly constant (Denlinger and Yokum 1998). Compared to that at the ambient temperature, hemolymph pH is decreased at lethal temperatures in *Centrioptera muricata* LeConte (Coleoptera: Tenebrionidae) (Ahearn 1970). With the increase in temperature, respiration rate is first increased and then decreased. It is uncertain whether this decreased respiration rate is an adaptation to conserve energy and to resume the normal metabolism in case the temperature decreases, or a result of a compromisation of metabolic activities due to temperature effect on the respiratory system. However, this decreased respiration rate is immediately followed by the death of insect (Neven 2000).

There are theories that describe the ultimate mechanism that causes insect death. The first one describes macromolecules (nucleic acid, protein, lipids) as the primary location of thermal wounding and death (Roti Roti 1982). The second theory proposes the cell membrane as the location for the initial thermal wounding, followed by death (Bowler 1987). However, in a multi-cellular organism, the susceptibility to heat increases with the complexity of the biological system. In that way, the order from least to most susceptible is as follows: macromolecules, cells, tissues, organisms (Prosser 1986).

Factors responsible for heat tolerance of insects

Insect survival at potentially lethal temperatures depends on a number of factors: temperature to which they are exposed, exposure period, species, thermal acclimation, relative humidity (Fields 1992; Burks et al. 2000; Beckett et al. 2007) and developmental stage (Davison 1969; Beckett et al. 2007). Insect heat tolerance is increased mainly by genetic adaptation, acclimation for a long period or the heat hardening that occurs within

a short period. As shown with *D. melanogaster*, insects from populations reared at relatively high temperatures over a few years survive under high temperature exposure better than populations reared at low temperatures (Cavicchi et al. 1995). Adults emerging from immatures reared at slightly higher temperatures show increased survival at high temperature (Levins 1969). Insect populations previously exposed to an intermediate high temperature for a short period survive more than the insects not exposed to that intermediate temperature, when exposed to a particular higher temperature (Levins 1969; Fields 1992).

There are several physiological mechanisms that confer heat tolerance; heat shock proteins are the most well-known. Under heat stress, the regular protein synthesis is suppressed and a new class of proteins called heat shock proteins is synthesized (Denlinger and Yokum 1998). Heat shock proteins are found in all prokaryotic and eukaryotic organisms (Feder and Hofmann 1999). Insects are capable of synthesizing HSPs (Singh and Lakhotia 2000; Lakhotia et al. 2002) when their body temperature rises above the optimum temperature for growth (Lurie and Jang 2007). Heat shock proteins are located in the cytoplasm or in organelles and are classified by the molecular weight. Among them, HSP 40 (40 kDa); HSP 60 (57-60 kDa); HSP 70 (69-71 kDa); HSP 80, 90 (80-94 kDa) and HSP 100 (100-114 kDa) are of high molecular weight. There are some other proteins termed small HSPs, with molecular weights between 15 and 45 kDa (Lurie and Jang 2007).

Under heat shock, the messages required for the synthesis of HSPs are transcriptionally regulated (Lindquist 1986). Heat shock proteins facilitate the proper function of the cell by acting as molecular chaperones and helping in a variety of

functions such as maintaining the integrity of newly-synthesized proteins (Vermeulen and Loeschcke 2007); preventing incompatible aggregation of denatured proteins, and properly refolding and stabilizing them (Parsell and Lindquist 1993; Becker and Craig 1994); and degradation of misfolded or aggregated proteins (Sonna et al. 2002; Morrow and Tanguay 2003). With the expression of HSPs, both the transcription of previously activated genes (Parsell and Lindquist 1993) and their translation (Lindquist 1986) are suppressed. In general, HSPs are expressed in response to stress conditions. In addition to their expression in response to heat, HSPs are also expressed with exposure to insecticides (Sharma et al. 2008), microbes (Ryu et al. 2008), denatured proteins (Parsell and Lindquist 1993) or cold (Joplin et al. 1990).

Some of the molecules that are synthesized in response to low temperature are also synthesized in response to high temperatures. *Bemisia argentifolii* Bellows and Perringor (Hemiptera: Aleyrodidae) exposed to 42ºC increases sorbitol levels compared to those maintained at 25ºC (Wolfe et al. 1998). This suggests a possible thermo-protective role by polyols. Furthermore, increases in the levels of tyrosine, alanine (Malmendal et al. 2006), dopamine (Rauschenbach et al. 1993) and trehalose (Thompson 2003) under stress conditions suggest that these compounds might be involved in the insect heat tolerance. Trehalose, the insect blood sugar, is generally a bio-stabilizing molecule (Argüelles 2000), and it plays a protective role in stabilizing proteins under heat stress (Thompson 2003). It is believed to maintain proteins partially folded under heat stress conditions and the cellular chaperones do the refolding, once the stress is removed (Singer and Lindquist 1998; Simola et al. 2000). When proteins are unfolded, trehalose is bound to proteins. Trehalose is released when proteins are returned to a folded state (Xie

and Timasheff 1997). Thus it helps to reactivate the proteins damaged by heat stress (Denlinger and Yocum 1998).

Heat tolerance of stored-product insects

Heat treatment is widely used in flour mills as a disinfestation method for stored-product insects (Fields and White 2002; Beckett et al. 2007). Therefore, it is important to characterize the heat tolerance of these insects, which varies among species. Many stored-product insects can survive "24 h at 40°C, 12 h at 45°C, 5 min at 50°C, 1 min at 55°C, and 30 s at 60°C" (Fields 1992, p. 108). Based on the survival of adults at 49°C, the relative heat tolerance from greatest to least is "*Lasioderma serricorne* (Fabricius) > *C. pusillus = R. dominica > S. oryzae = Tribolium castaneum = Trogoderma variable* Ballion > *S. granarius = Gibbium psylloides* (Czenpinski) > *Cathartus quadricollis* (Guerin-Meneville) = *O. mercator > Tribolium confusum= O. surinamensis*" (Fields 1992, p. 108).

In stored-product insects, acclimation at temperatures 35-40°C increases the survival at temperatures up to 55°C (Fields and White 2002). Pre-exposure at 32 or 35°C increases the survival at 40°C in *S. oryzae* and in *S. granarius*. The maximum acclimation is obtained in *S. granarius* with 14 days at 32°C or 2 days at 35°C, whereas in *S. oryzae* acclimation is most effective with 2 days at 32°C or 1 day at 35°C. Longer exposures tend to reverse the obtained acclimation (Gonen 1977).

In *T. castaneum*, generally young larvae are the most tolerant to temperatures between 50-60ºC (Mahroof et al. 2003). The expression of HSP 70 in young larvae of *T. castaneum* under heat stress has been detected (Mahroof et al. 2005c), and the genes

responsible for this expression have been identified: *tchsp 70 I*, *tchsp 70 II* and *tchsp 70 III* (Mahroof et al. 2005b). In response to heat shock at 40°C, expression of HSP70 I is increased, HSP70 II is constitutive and HSP70 III differs among developmental stages (Mahroof et al. 2005b).

Fecundity and fertility

Factors affecting insect fecundity/fertility

Fecundity is the total number of eggs produced by a female (Southwood 1978). Fertility is the total number of live eggs (Southwood 1978) or the number of larvae hatching (Park et al. 1958). Fecundity and fertility are affected by the number of spermatophores and by the frequency of mating (Butlin et al. 1987; Walker and Allen 2010). In some butterflies, males transfer sodium to the female during copulation, and this increases fertility (Pivnick and McNeil 1987).

Hormonal impacts on the female and male reproduction

Juvenile hormone affects spermatogenesis (Koeppe et al. 1985; Ashok 2000), production of male-accessory-gland proteins (Gillott and Gaines 1992; Gillott 1996), communication for mating (Teal et al. 2000) and mating (Segura et al. 2009). Removal of corpora allata (reduction of JH) during the pre-adult stages increases spermatogenesis in *P. americana* (Blaine and Dixon 1970). The effects of JHAs on spermatogenesis vary from species to species. For example, JHAs reduce spermatogenesis in *Rhodnius prolixus* Stål, (Hemiptera: Reduviidae) (Dumser and Davey 1974), but increase spermatogenesis in *Pterostichus nigrita* (Paykull) (Coleoptera: Carabidae) (Ferenz 1963). Developing

male accessory glands in *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae) larvae are more sensitive to JH than are the germinal tissues. In this species, JH has to be present during the fourth instar larvae for successful maturation of accessory glands and cannot be replaced at a later stage, proposing "the existence of a JH-dependent juvenile phase" (Koeppe et al. 1985; p. 187). In *Spodoptera mauritia* (Boisduval) (Lepidoptera: Noctuidae), treatment with an ecdysone analogue increases spermatogenesis (Benny and Nair 2006). In *T. castaneum*, JH deficient males have a reduction in mating behavior and sperm transfer. Furthermore, mating of such males with normal females reduces fecundity (Parthasarathy et al. 2009).

There is information about the role of JH in reproduction of female insects. JH stimulates vitellogenesis in the fat body in cockroach *Blattella germanica* (L.) (Dictyoptera: Blattellidae) (Comas et al. 1999). In *Drosophila*, both JH and ecdysteroids are necessary for vitellogenesis (Bownes 1989; Richard et al. 2001). In *Blaberus discoidalis* Audinet-Serville (Dictyoptera: Blaberidae), JH induces ovarian protein synthesis (Keeley and McKercher 1985). In female *T. castaneum*, JH increases vitellogenesis (Parthasarathy et al. 2010b), and ecdysteroid stimulates growth of ovaries and maturation of primary oocytes (Parthasarathy et al. 2010a). In the stick insect *Dixippus morosus* Br. (Orthoptera, Phasmidae), development of eggs and oviducts is decreased when the late-instar nymphs are provided with JH (Koeppe et al. 1985). In female *P. americana*, JH applied at the nymphal stage reduces the development of the colleterial gland (Bodenstein and Sprague 1959).

Insect growth regulators and fecundity

Juvenile hormone analogues have demonstrated their effects on the development of male and female reproductive systems, which subsequently affects their reproduction as adults. Exposing male or female larvae of Western spruce budworm *Choristoneura occidentalis* Freeman (Lepidoptera: Tortricidae) to methoprene (ZR515) or hydroprene reduces adult fecundity (Robertson and Kimball 1979). In some cases, the effects of JHAs depend on the life stage. Methoprene applied to *C. pomonella* fifth instar female larvae aged 2 and 6 days, reduces fecundity. However, there is no reduction in fecundity when 4-day-old larvae are exposed to methoprene (Brown and Brown 1982).

Sex-specific effects of methoprene are detected in certain species. When male and female larvae of the summer fruit tortrix moth *Adoxophyes orana* (Fischer von Roslerstamm) (Lepidoptera: Tortricidae) are exposed to JHA (Ro 10-3108/018), fecundity of adults is reduced. In this case, females are affected more than males, as indicated by the number of eggs produced when paired with an untreated mate (Schmid et al. 1978).

Fecundity and fertility of stored-product insects and the effects of juvenile hormone/ juvenile hormone analogues

The average fecundity of a *T. castaneum* female is 360 eggs at 25°C and 70% r.h. (Howe 1962). The oviposition rate of *T. castaneum* is relatively constant at 2.5 eggs/ day for the first 3 months, after which there is a gradual decline in fecundity. Both low and high temperatures reduce fecundity. At 20°C, the fecundity is as low as one egg for 15 females/ day (Howe 1962). Also, the heat shock affects the reproduction of *T. castaneum*.

Fecundity, reproductive fitness and progeny production by emerged adults are all reduced when parents are exposed to 50-60°C for 39 min as pupae or adults. These effects are greater when the pupae are exposed, and females are affected more than males (Mahroof et al. 2005a). In *T. castaneum*, the probability of producing offspring is not affected by number of matings, when females are mated with virgin males. However, when mated with non-virgin males, female fertility is increased with multiple matings (Pai et al. 2005). Fecundity of *T. castaneum* is affected by food. Fecundity is highest in flour, followed by cracked maize and the lowest in undamaged maize (Li and Arbogast 1991). Fecundity of F_1 *T. castaneum* females reared in the laboratory is lower than that of wild parents (Daglish 2005). This may be due to the differences in the developmental periods and/or pupal weight between the two populations, as described by White (1984).

Adults of *R. dominica* (Allanson and Wallbank 1994; Daglish and Pulvirenti 1998; Chanbang et al. 2008) and *O. surinamensis* (Allanson and Wallbank 1994) exposed to methoprene reduce fecundity. Exposure of *R. dominica* eggs to methoprene reduces their fertility (Chanbang et al. 2008). Progeny production by adult cigarette beetles *L. serricorne* is decreased with increased exposure to methoprene. There is no effect on the reproductive behaviour; the disruptive effects on reproduction occur during embryonic development (Marzke et al. 1977). When the eggs of *P. interpunctella* are exposed to *Cecropia* JH, the mating frequency of the resulting males and the number of spermatophores transferred to females is reduced with increased JH concentrations, although responsiveness of males to female sex pheromone is not affected (Oberlander et al. 1975).

When *T. castaneum* males and females are exposed to synthetic JH or JHAs, fecundity is not affected; however, fertility is reduced in the majority of treatments (Williams and Amos 1974). Exposure of female adults of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) to hydroprene does not reduce fecundity but reduces fertility (Rup and Chopra 1984). Therefore, there is a research gap to explore how the differential effects of JHAs on fecundity and fertility would affect the progeny production of *T. castaneum*.

Residual efficacy of stored-product insecticides

Residual insecticides are applied as liquids or dusts to storage structures or directly to commodities. Residual insecticides offer several advantages, such as persistence, broad-spectrum activity and convenience in application (Hagstrum and Subramanyam 2006). The use of residual insecticides with different modes of action helps to suppress resistance development of insects (Hagstrum and Subramanyam 2006). Residual insecticides are used with other control methods in IPM. There are a number of factors that affect the efficacy of a residual insecticide (Snelson 1987): type of insecticide (Arthur 1996), formulation (Arthur 1996), application method (Arthur 1997a), composition of the treated surface (Arthur 1997a; Toews and Subramanyam 2003), cleanliness of the surface (Watters 1970; Toews et al. 2010), temperature (Hadaway and Barlow 1957), duration of exposure (Arthur 1997b; Arthur 1998a) and insect species (Sparks and Hammock 1982; Arthur 1997a; Toews et al. 2003).

The residual efficacy of several insecticides has been tested against *T. castaneum*. Cypermethrin, fenvelerate or permethrin applied on plywood surfaces has diminishing efficacy on *T. castaneum* adults over a 33-week period at 30ºC (Watters et al. 1983). Generally, storage at low temperatures increases persistence of insecticides (Abdel-Kader et al. 1980; Abdel-Kader et al. 1982). Malathion or fenitrothion residues decline less after 72 weeks of storage on wheat at −35ºC than at 27ºC (Abdel-Kader et al. 1980; Abdel-Kader et al. 1982). Wheat treated with malathion (Strong and Sbur 1960) or diazinon (Strong and Sbur 1965) has reduced efficacy against *S. oryzae* after storage at higher temperatures. Furthermore, degradation of bromophos on wheat is faster at 26ºC than at 15ºC (Eichler and Knoll 1974).

The effect of methoprene has been tested on surfaces and grains. Methoprene (Altosid SR10), applied on jute sacks, prevents adult emergence of *Ephestia cautella* (Walker) (Lepidoptera: Phycitidae) (Gonen and Schwartz 1978). Development of *E. cautella* larvae into adults is lower on paper surfaces treated with methoprene and maintained at 18ºC than on those held at 26ºC (Gonen and Schwartz 1978). Both methoprene (Altosid ZR515) and hydroprene (Altozar ZR512) applied on cardboard surfaces have reduced efficacy (prevention of adult emergence) against *E. kuehniella* with increased duration at 30ºC (Tan and Tan 1980).

There have been some studies on the persistence of JHA on grains over a few months (Hoppe and Suchy 1975). Storage at 27ºC of wheat treated with methoprene at 1-10 ppm arrests the development of *R. dominica* and *S. oryzae* for 12 months after application (Mian and Mulla 1982b). Methoprene also shows suppression of insect development, after 28 weeks on maize at approximately 30ºC (Daglish et al. 1995), and

over 6.5 months on sorghum at 22-28ºC (Daglish and Wallbank 2005). A GC analysis also confirms the presence of methoprene residues on wheat, held at 27ºC for 12 months following application (Mian and Mulla 1983).

Chapter 3. Effect of methoprene on the heat tolerance and cold tolerance of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

Modified from: Wijayaratne, L.K.W., Fields, P.G., 2010. Effect of methoprene on the heat tolerance and cold tolerance of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Journal of Stored Products Research 46: 166-173 (doi: 10.1016/j.jspr.2010.04.001).

Abstract

Methoprene, a juvenile hormone analogue, was evaluated for its ability to alter heat tolerance or cold tolerance of *Tribolium castaneum*, the red flour beetle. Young adults and late-instar larvae were exposed to a series of methoprene concentrations. They were held either at 46°C or 0°C for different durations, and survival of adults or the adult emergence from larvae was recorded to determine their tolerance to extreme temperatures. At 46°C, the time to kill 50% of the population (95% CL) for untreated adults was 10.8 (9.6-11.8) h compared to 9.3 (8.3-10.0) h for adults exposed to 3.33 ppm of methoprene for 48 h. Higher concentrations of methoprene also caused adults to be less heat tolerant. In contrast, there was no evidence that methoprene reduced the heat tolerance of larvae. At 0°C, both unacclimated and cold-acclimated insects were tested. Methoprene did not affect the cold tolerance of adults or larvae, regardless of cold-acclimation. As seen in other studies, methoprene was not toxic to adults even at 66.6 ppm, and it was highly toxic to larvae (LD_{50}=0.015 to 0.020 ppm). Cold tolerance was slightly greater in both adults and larvae, after being held at 15°C for two weeks. This is

the first study to report that a juvenile hormone analogue has an impact on insect heat tolerance.

Keywords: adult, larvae, Diacon, juvenile hormone, synergy

Introduction

The infestation of insects in stored products dates back to early civilization (Cotton 1963; Sokoloff 1972). The red flour beetle, *Tribolium castaneum*, has long been reported as a major insect pest of both raw and processed forms of grains (Mickel and Standish 1947; Daniels 1956; Champ and Dyte 1976; Li and Arbogast 1991). This insect is found in warehouses (Mullen 1992), flour mills (Zettler 1991), processing plants (Campbell et al. 2002) and retail stores (Arbogast et al. 2000).

Current control measures for *T. castaneum* or other stored-product insects include the use of contact insecticides such as the pyrethroid cyfluthrin (Arthur and Dowdy 2003), application of diatomaceous earth (Dowdy 1999b), fumigation with phosphine (Rajendran 2000) or sulfuryl fluoride (Reichmuth et al. 2003), use of low temperature (Fields 1992) and high temperature (Arthur 2006; Beckett et al. 2007). There are concerns with the use of synthetic chemicals to control stored-product insects, including the presence of residues in food, resistance development by pest species, health risks (Arthur 1996), increased cost (Hagstrum and Subramanyam 2006) and toxicity to non-target organisms (Fields 1992).

Methoprene is an IGR that is a JHA (Chanbang et al. 2008), disrupting the development of larvae to adults. JHAs do not kill adults (Oberlander and Silhacek 2000),

however, methoprene can have an indirect impact on adults by reducing their fecundity (Daglish and Pulvirenti 1998; Brown and Brown 1982). The effects of methoprene on stored-product insects have been extensively studied (Loschiavo 1976; Samson et al. 1990; Oberlander et al. 1997; Arthur 2004). Methoprene was registered as an insecticide, Diacon, in the U.S.A. in 1980s and contained both the R- and S-isomers (Arthur 2004). It was reintroduced in 2002, with only the biologically active S-isomer of methoprene, by Wellmark International with their product Diacon II (Chanbang et al. 2008). In the U.S.A., it is registered for direct application to grains, as a contact insecticide or as an aerosol application (Jenson et al. 2009). In Canada, methoprene was first registered in 1977 for mosquito control (Health Canada 2001).

Heat was used as early as in 1700 in France to control *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) in stored wheat (Duhamel du Monceau and Tillet 1762). It was used to control stored-product insects in flour mills as early as at the beginning of the twentieth century (Dean 1911). There has been a renewed interest in heat to control insects because of the phase-out of methyl bromide (Fields and White 2002; Beckett et al. 2007). During structural heat treatments, temperatures of 50-60°C are maintained for 24-36 h to control all stored-product insects (Fields and White 2002). However, there are some limitations to using heat as an insect pest management strategy, including the increased cost compared to other control methods (Dosland et al. 2006), damage to sensitive equipment (Dowdy and Fields 2002) and uneven distribution of heat (Dowdy 1999a). Therefore, it would be useful to determine if heat could be used with other treatments to make it more effective.

There are several examples of controlling stored-product insects by combining more than one treatment, to produce more effective control than each treatment used in isolation (Banks 1987; Banks and Fields 1995). Some of these include the combinations of low temperature and ice-nucleating-active bacteria (Fields 1993), high temperature and diatomaceous earth (Dowdy and Fields 2002), pea protein and parasitoids (Hou et al. 2004), the organophosphate chlorpyrifos-methyl and the pyrethroid cyfluthrin (Arthur 1994b), and the use of heat, carbon dioxide and phosphine (Mueller 1994). Methoprene has been used in combination with other IGRs (Daglish and Wallbank 2005; Yonggyun and Krafsur 1995) or with other insecticides (Edward et al. 1993; Daglish 2008). It is more meaningful to seek using methoprene in combination with other control methods, as IGRs are considered to be better used in an integrated pest management program, rather than it being used alone (Oberlander et al. 1997).

Most studies with methoprene and stored-product insects have focused on the effects on development or mortality. The effect of juvenile hormone or its analogues on extreme temperature tolerance of insects is not well-studied. A few studies tested the effect of hormones on cryoprotectants and other molecules at freezing temperatures (Tsumuki and Kanehisa 1980; Horwath and Duman 1983; Hamilton et al. 1986; Xu et al. 1990). However, none of these studies examined how hormonal treatments affect the survival of insects at freezing temperatures. Although cold acclimation, in which exposure to an intermediate temperature prior to the exposure at the target low temperature increases insect cold tolerance is well documented (Fields 1992), there has been no attempt to explore the potential effect of hormones on cold acclimation. This is the first study to explore the synergistic effect of a JHA in combination with increased

temperatures. The objectives of this study were to determine if the JHA methoprene affects the ability of *T. castaneum* adults or larvae to tolerate high or low temperatures and if methoprene affects cold acclimation.

Materials and methods

Methoprene

Diacon II (Central Life Sciences, Schaumburg, Illinois, U.S.A.) was used as the source of methoprene. *Tribolium castaneum* adults or larvae were exposed to a series of methoprene concentrations in heat and cold tolerance experiments. To ensure that any effects were caused by methoprene, the adjuvant mixture of the Diacon II containing all components except methoprene was used at the highest concentration used for methoprene. All trials had two controls: distilled water and adjuvants. All the solutions were prepared immediately before spraying.

Spraying was carried out in a fume hood using an artist's brush (Model H#1L, Paasche Airbrush Company, Chicago, U.S.A.). The air pressure of the sprayer was maintained constant (103 kPa) throughout the experiment. Spraying was done in the following order: distilled water, adjuvants and finally the methoprene solutions starting from the lowest concentration to the highest concentration. Furthermore, the new concentration was passed through the sprayer, before the wheat sample was sprayed with that particular concentration. The above two steps were adopted to avoid contamination from a higher concentration and to avoid carry over of residues from the previous lower concentration, respectively. Hard red spring wheat *Triticum aestivum* L. (14.0-14.4% moisture content) containing 80% whole wheat and 20% cracked wheat was sprayed with

the treatments. A 300 g wheat sample was spread in a single-kernel layer on waxed paper. There were four replicates in each experiment. From each concentration, 3 mL was placed in the reservoir of the artist's air brush, and was sprayed on to the 300 g grain sample. This 300 g sample sprayed with a particular concentration was used to serve all durations of holding with one replicate. Spraying the four replicates of the same concentration was done consecutively. Immediately following spraying, each grain sample was hand tumbled for 30 s (Arthur 2004) in a plastic bag for uniform dispersion of the compounds in the grain sample. A new waxed sheet was used for the spraying of each 300 g sample of wheat. Glass vials, each of 35 mL and covered with a cap with a wire mesh insert for aeration, were each filled with 20 g of the treated wheat.

Insects

Tribolium castaneum used in the experiment originated from farm grain bins near Steinbach, Manitoba and had been cultured in the laboratory since 1989. Insects were reared at 30°C, 70% r.h., in the dark in a medium containing 95% unbleached white wheat flour and 5% brewer's yeast (ICN Biomedicals, Inc., Aurora, Ohio, U.S.A.). The progeny adults and larvae used in heat tolerance and cold tolerance experiments were produced by allowing two hundred parent adults to lay eggs on 250 g of the above medium for 3 d. Adults were 9-14 days of age since emergence and of mixed sex, and late-instar larvae were 10-12 days from hatching, when they were placed on the treated wheat.

Tribolium castaneum cultures were sieved through an 850 µm mesh sieve to separate the 9-14 d old adults. The adults were moved into a vial using a vacuum line to

minimize handling. To separate the 10-12 d old larvae, the rearing media were sifted through a 425 μm mesh sieve. A reference size for larvae was used to ensure using approximately the same-sized larvae in the experiment. The larvae were scooped on to a piece of paper, and were gently introduced into the vial containing the sprayed wheat. Twenty adults or larvae were introduced into each vial earlier filled with 20 g of treated wheat. These vials were held at 30°C, 70% r.h. and in total darkness for 36-48 h, before being subjected to heat or cold treatments.

Heat tolerance experiment

In the experiments with adults, wheat was treated with 1.67, 3.33, 16.65, 33.3 or 66.6 ppm of methoprene (w/w). For larvae, methoprene concentrations of 0.003, 0.00825, 0.0165, 0.033 or 0.066 ppm were used based on preliminary experiments, to have a range of no effect to 100% mortality. There is 288 g of methoprene in 1 L Diacon II. Various volumes of Diacon II were dissolved in 1 L of distilled water so that 3 mL sprayed on 300 g of wheat would give different concentrations. Water and adjuvants, measured as equivalent amount of methoprene used at the highest concentration, were used as controls; water and 66.6 ppm of adjuvants for adults, water and 0.066 ppm of adjuvants for larvae.

Heat treatments were carried out in an oven at 46±0.5°C (Thermocenter TC40/ TC100, Salvis Lab, Rotkreuz, Switzerland). For adults, the exposure periods were 0, 6, 9, 11, 13, 15 or 17 h. For larvae they were 0, 3, 6, 9, 12, 15 or 18 h. Temperature was measured by placing thermocouples (HOBO data loggers, Onset Computer Corporation, MA, U.S.A.) at the centre of wheat in the vials placed at the front and back of shelves in

the oven. It took 89-126 min in the experiment with adults and 132-193 min in the experiment with larvae to reach 46°C, after the vials were placed in the oven. Each time a group of vials were removed from the oven, there was a drop of 2-3°C with adults or 0.3-2.0°C with larvae. It took approximately 60-90 min with the adults or 10-60 min with larvae for temperature to regain 46°C. The warm-up periods and drops in temperature were not included in the total time taken as at 46°C. Following heat treatments, the vials containing adults or larvae were placed in an incubator maintained at 30°C and 70% r.h. The survival of adults was determined within 24 h from the termination of exposure at 46°C. Effect on larvae was determined as adult emergence and was done 3 weeks after the end of heat exposure.

Cold tolerance experiment

Methoprene concentrations used were the same as for the heat tolerance experiment except the 0.066 ppm treatment of larvae was not used. The controls were used as described above in the heat tolerance experiment. Following the incubation period of 36-48 h at 30°C, one set of vials containing treated grain and insects was exposed directly to 0±0.2°C and 65±10% r.h. After the incubation period, the second set of vials was first transferred to a growth cabinet at 15°C and 60-70% r.h., total darkness for two weeks for cold acclimation and was then exposed to 0°C. The vials containing insects were first covered by a polythene cover and then placed inside a portable cooler containing crushed ice. The cooler was placed at 2.5°C and thus the vials were exposed to the temperature of melting ice. The temperature inside vials was measured using HOBO data loggers.

Tribolium castaneum adults were exposed for 0, 3, 4, 5, 6 or 7 days if unacclimated, with additional treatments for 8 or 9 days if acclimated. The larvae were exposed to 0°C for 0, 2, 3, 4, 5 or 6 d if unacclimated, with an additional treatment for 8 d if acclimated. Upon the completion of these different periods at 0ºC, the vials were transferred to the growth cabinet at 30ºC with 60-70% r.h., and total darkness. After three weeks, the adults were counted.

Data analysis

The experimental design for the heat tolerance experiment was a complete randomized two-factor factorial design. The first factor was the different methoprene concentrations and the second factor was the duration of exposure (h) to the elevated temperature, 46°C. The vials were considered replicates. The experimental design for the cold tolerance experiment was a complete randomized three-factor factorial design. The first factor was the different methoprene concentrations, the second factor was the duration of exposure (days) to 0°C and the third factor was the cold acclimation at 15°C. As in heat tolerance experiment, vials were considered replicates.

All means in the text are given with standard errors of mean. The proportion of surviving adults was transformed by taking the square root of the arcsine and was analyzed using ANOVA procedures of Statistical Analysis System (SAS) (SAS Institute 2002-2008). Means of survival were separated by Tukey's test with the significance level of 0.05, for a particular duration of exposure to 46°C in the heat tolerance experiment or for a particular duration of exposure to 0°C, either in unacclimated or cold-acclimated batch of insects, in the cold tolerance experiment. Probit analysis used the probit

transformation of mortality, the $\log_e$ transformation of concentrations and adjusting for control mortality with the natural response parameter (PoloPlus, LeOra Software). For a given concentration, the LT_{50} (95% CL) was determined. Comparison of LT_{50} was done using the lethal ratios (Robertson and Priesler 1992). The lethal ratio at 50% mortality (LT_{50} ratio) for a given concentration was determined as LT_{50} for adjuvants/LT_{50} for a particular concentration.

Results

Heat tolerance of adults

Significant differences in the survival of adults occurred with methoprene concentration (ANOVA, $F_{6,147}$=30.39, P<0.0001), duration of exposure (h) (ANOVA, $F_{6,147}$=942.11, P<0.0001) and the interaction of methoprene concentration x duration of exposure (ANOVA, $F_{36,147}$=11.09, P<0.0001).

For the *T. castaneum* adults, there was no effect of methoprene alone at 0 h exposure to 46°C (Table 3.1). In general, there was no difference between the two controls, water and adjuvants, on the survival of adults (ANOVA, $F_{1,42}$=0.15, P=0.7010) except at 11 h exposure, where the survival of adults exposed to adjuvants was lower than the water control.

Increased exposure to 46°C caused reduced survival of adults. In general, pre-exposure to methoprene synergistically reduced the survival of *T. castaneum* adults compared to the controls with water or adjuvants. This is evident with 6, 9, 11 or 13 h exposures at 46°C, when the means for survival of adults were separated by Tukey's test. There are certain outliers possibly due to variation of the heat in the oven. The LT_{50} ratios

at 3.33 ppm or higher concentrations showed significant differences compared to the adjuvants. The LT_{50} ratio for water was slightly lower than adjuvants (0.92), may be due to the sudden drop of survival in the adjuvants at 11 h exposure. However, over and above the behavior of those outliers, the results show that pre-exposure to methoprene made *T. castaneum* adults more sensitive to heat.

Heat tolerance of larvae

There were significant differences in adult emergence following larval treatment due to methoprene concentration (ANOVA, $F_{6,147}$=242.73, P<0.0001), duration of exposure (h) (ANOVA, $F_{6,147}$=17.87, P<0.0001) and the interaction of methoprene concentration x duration of exposure (ANOVA, $F_{36,147}$=4.27, P<0.0001).

With no exposure to heat (0 h), methoprene was toxic to *T. castaneum* larvae with LD_{50} (95% CL) of 0.020 (0.017-0.024) ppm methoprene, and almost complete suppression at 0.066 ppm (Table 3.2). With increased exposure at 46°C, there was no difference between the water control and adjuvants (ANOVA, $F_{1,42}$=0.15, P=0.9231), indicating that any effects in larvae exposed to methoprene concentrations were due to either methoprene or the heat. Adult emergence in the larvae treated with 0.003 and 0.00825 ppm methoprene were not different from controls (water or adjuvants), with no heat (0 h). Furthermore, when larvae were exposed to heat, there was no consistent difference between controls and these two lowest concentrations except with 0.003 ppm at 15 h exposure, according to Tukey's mean separation test.

For the controls, the adult emergence ranged from 98.8-100% at 0 h, which reduced only to approximately 50% at the longest exposure time, 18 h (Table 3.2). Given

that the two lowest methoprene concentrations did not show significant differences from the controls both at 0 h exposure and at increased exposure periods and that the LT_{50} ratios for those two concentrations were not different from that of the adjuvants, I conclude that methoprene did not make larvae more sensitive to heat. Methoprene alone had a strong impact on larvae to prevent their development to adults, as evident at the three higher concentrations without any exposure to heat (0 h). This made it impossible to calculate the LT_{50} due to the high mortality at 0 h (Table 3.2). Therefore at exposures greater than 0 h at 46°C, it is difficult to isolate the effects caused by the combination of heat and methoprene from those of methoprene alone. It may be possible that methoprene also reduced the heat tolerance of *T. castaneum* larvae, as in adults, but this reduction in survival (emergence to adults) was too small to be detected against the huge effects of methoprene alone on larvae.

Cold tolerance of adults

Significant differences in the survival of adults occurred with the acclimation (ANOVA, $F_{1,324}$=677.51, P<0.0001), methoprene concentration (ANOVA, $F_{6,324}$=4.2, P=0.0004), duration of exposure (days) (ANOVA, $F_{7,324}$=234.24, P<0.0001), and interaction of acclimation x duration of exposure (days) (ANOVA, $F_{5,324}$=73.23, P<0.0001). In contrast, there were no significant differences in the interactions of acclimation x methoprene concentration (ANOVA, $F_{6,324}$=2.09, P=0.054) and methoprene concentration x duration of exposure (ANOVA, $F_{42,324}$=0.90, P=0.6481).

As in the heat tolerance experiment, methoprene alone (0 d) was not toxic to *T. castaneum* adults, in both unacclimated and in cold-acclimated groups. However,

significant differences between the survival of adults treated with water or adjuvants, were detected both in unacclimated adults (ANOVA, $F_{1,36}$=11.96, P=0.0014) and in cold-acclimated adults (ANOVA, $F_{1,48}$=9.93, P=0.0028), according to Tukey's mean separation test and based on the LT_{50} ratios (Table 3.3). This was due to the significant differences in 3 day exposure in the unacclimated batch (ANOVA, $F_{1,6}$=13.93, P=0.0097) and 6 day (ANOVA, $F_{1,6}$=9.74, P=0.0206), 7 day (ANOVA, $F_{1,6}$=12.42, P=0.0124), and 9 day exposure (ANOVA, $F_{1,6}$=39.3, P=0.0008) in the cold-acclimated batch.

Survival of adults decreased with the increase in the duration at 0°C. By acclimating adults at 15°C for two weeks, the cold tolerance of *T. castaneum* adults was increased; in the controls, LT_{50} (95% CL) increased from 3.59 (3.29-3.77) d for unacclimated adults to 6.02 (5.39-6.46) d for cold-acclimated adults. Furthermore, the LT_{50} ratio (95% CL) for cold-acclimated vs. unacclimated adults treated with adjuvants was 1.84 (1.71-1.97) showing a significant increase in cold tolerance due to acclimation. However, neither the unacclimated nor cold-acclimated adults showed pronounced and consistent reduction in the LT_{50} between the controls and the methoprene-treated adults. This is evident from the LT_{50} ratio values, too. Therefore, methoprene has not affected the cold acclimation of *T. castaneum* adults. Furthermore, absence of significant differences in the survival of adults in the controls and in the methoprene treatments for a particular duration of exposure, either in unacclimated or cold-acclimated batch, shows that methoprene does not make *T. castaneum* adults more sensitive to cold.

Cold tolerance of larvae

There were significant differences in the emergence of adults resulting from acclimation (ANOVA, $F_{1,259}$=77.91, P<0.0001), duration of exposure (days) (ANOVA, $F_{6,259}$=165.98, P<0.0001), methoprene concentration (ANOVA, $F_{5,259}$=152.91, P<0.0001), acclimation x duration of exposure (ANOVA, $F_{5,259}$=15.51, P<0.0001), acclimation x methoprene concentration (ANOVA, $F_{5,259}$=7.30, P<0.0001), and methoprene concentration x duration of exposure (ANOVA, $F_{30,259}$=7.92, P<0.0001).

As in the heat tolerance experiment, methoprene was highly toxic to *T. castaneum* larvae with no exposure to cold (0 d) (Table 3.4). The LD_{50} (95% CL) for both unacclimated and cold-acclimated larvae with no exposure to cold (0 h) was 0.015 (0.012-0.018) ppm. There were no significant differences between water and adjuvants in terms of adult emergence for any of the exposure periods, either in unacclimated larvae (ANOVA, $F_{1,36}$=0.30, P=0.5879) or cold-acclimated larvae (ANOVA, $F_{1,42}$=2.95, P=0.0935), according to Tukey's mean separation test. The LT_{50} ratio showed a slight difference between the two controls in the unacclimated larvae (Table 3.4).

Survival of larvae decreased with increased duration of exposure to 0°C. As in the adults, there was an increase in the cold tolerance after larvae were held at 15°C for two weeks before the cold exposure at 0°C. In the water controls, cold-acclimated larvae had LT_{50} (95% CL) of 4.42 (3.61-5.09) days compared to 2.85 (2.39-3.20) days for unacclimated larvae. The LT_{50} ratio (95% CL) for cold-acclimated vs. unacclimated larvae treated with adjuvants was 1.42 (1.21-1.66) showing a significant increase in cold acclimation in the controls. There were no consistent differences in LT_{50} values between the controls and the methoprene-treated larvae either in unacclimated or cold-acclimated

treatments, according to Tukey's mean separation test. The LT_{50} ratio values also show that. Therefore, as in the case of cold tolerance of adults, methoprene did not affect the cold acclimation of larvae. Furthermore, it did not affect cold tolerance of larvae at 0°C.

Discussion

Methoprene reduced the heat tolerance of *T. castaneum* adults. The death of stored-product insects at increased temperatures occurs due to changes in the membrane lipids, rate imbalances of biochemical reactions, disturbance in the ionic activities or due to desiccation (Fields 1992; Denlinger and Yokum 1998). Insects undergo a wide array of physiological changes in response to heat stress to mitigate these adverse effects. These include synthesis of heat shock proteins (Lurie and Jang 2007); increased production of the insect blood sugar trehalose (Singer and Lindquist 1998); increased levels of glycerol or sorbitol (Denlinger and Yocum 1998; Wolfe et al. 1998), amino acids (Malmendal et al. 2006) or dopamine (Rauschenbach et al. 1993) in the body. One or more of these protective processes may have been adversely affected by methoprene.

Among the many factors responsible for heat tolerance, heat shock proteins are the most well-known (Denlinger and Yocum 1998). Insects synthesize heat shock proteins when their body temperature rises above the favorable temperature for the growth (Lurie and Jang 2007). Heat shock proteins stabilize denatured proteins (Parsell and Lindquist 1993), prevent deleterious aggregations of proteins (Parsell and Lindquist 1993; Becker and Craig 1994) and degrade misfolded or aggregated proteins (Sonna et al. 2002; Morrow and Tanguay 2003). Heat shock protein 70 is induced in *T. castaneum* larvae, pupae and adults when exposed to 40°C (Mahroof et al. 2005c). As only the heat

tolerance of adults was reduced by methoprene and not that of larvae of *T. castaneum,* it is possible that prior exposure to methoprene may perturb the expression of a specific heat shock protein that is present only in adult *T. castaneum*, but not in larvae. There is support for the hypothesis that methoprene triggers this kind of interference. Heat shock protein synthesis is transcriptionally regulated (Lindquist 1986) and JH acts within the nucleus (Riddiford 1994), so could regulate the synthesis of heat shock proteins. In *Drosophila*, JH III and methoprene suppress the expression of small heat shock protein genes hsp 22 and hsp 23 mediated by 20-hydroxyecdysone (Berger et al. 1992). In *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) pupae, synthesis of higher levels of stress protein dsp 28 is induced by methoprene (Graham et al. 1996). Therefore, it is possible that exposure to methoprene prior to heat treatment affected the heat shock protein synthesis in *T. castaneum* adults and reduced their heat tolerance.

One way to test the hypothesis that JH reduces the heat tolerance of *T. castaneum* adults would be to treat insects with the juvenile hormone antagonist, precocene, which should decrease endogenous JH. From the hypothesis, I predict that the decrease of JH titer in the insect body would increase heat tolerance in adults, and have no effect in larvae. Another, but technically more difficult experiment would be to measure JH levels (Cusson et al. 1994) in insects during exposure to heat. I expect lower JH levels in response to heat in surviving adults.

Methoprene had no effect on cold tolerance of *T. castaneum* larvae or adults. This means that methoprene did not affect the physiological and biochemical processes that induce cold tolerance. Insect cold tolerance is induced by several mechanisms. In freeze-intolerant species, these include elimination of ice nucleators from the body (Baust

and Zachariassen 1983; Boiteau and Coleman 1996), synthesis of molecules such as low-molecular-weight cryoprotectants (Ishiguro et al. 2007) or thermal-hysteresis proteins (anti-freeze proteins) (Tyshenko et al. 1997), and depression in the hemolymph SCP (Duman 1982). In addition, methoprene did not affect cold acclimation. In *T. castaneum*, mean chill-comma temperature is lowered by acclimation at 15ºC for two weeks (Evans 1981). This is in agreement with the findings of the current research.

There are only a few studies on hormonal effects on cold tolerance. Application of JH or JHAs affects larvae: JH increases the anti-freeze protein levels in the larvae of *D. canadensis* (Horwath and Duman 1983; Xu and Duman 1991); increases glycerol and sorbitol levels in the 3rd instar larvae of the gall fly *E. solidaginis* (Hamilton et al. 1986); increases trehalose level in *S. lurida* (Cho et al. 2007); reduces trehalose levels in *Anthonomus pomorum* (L.) (Coleoptera: Curculionidae) (Zdarek et al. 2000); lowers the SCP in the larvae of *E. solidaginis* (Rojas et al. 1987); depresses the SCP in freeze-sensitive beetle species such as *Melanotus rufipes* (Herbst) (Coleoptera: Elateridae), *Platycerus caprea* (De Geer) (Coleoptera: Lucanidae), *Xylita laevigata* (Hellenius) (Coleoptera: Melandryidae), *Zilora ferruginea* (Paykull) (Coleoptera: Melandryidae) and *Bolitophagus reticulatus* (L.) (Coleoptera: Tenebrionidae) (Zachariassen 1980); induces ice nucleators in *C. suppressalis* (Tsumuki and Hirai 1999); reduces ice nucleators and SCP in the larvae of the stag beetle *C. piceus* (Xu et al. 1990); and terminates cold tolerance in *Aulacophora nigripennis* Motschulsky (Coleoptera: Chrysomelidae) (Watanabe and Tanaka 2000). Juvenile hormone is involved in maintaining the diapause in some species with a larval diapause such as *Diatraea grandiosella* Dyar (Lepidoptera: Pyralidae) (Chippendale and Turunen 1981). In some other species such as *Ostrinia*

nubilalis (Hübner) (Lepidoptera: Crambidae), JH may not interfere with maintaining diapause status although it may regulate diapause induction (Chippendale and Yin 1979). Diapause and cold hardiness can be linked, especially in species that have an overwintering diapause (Denlinger 1991). However, there are insects in which the diapause and cold hardiness are not linked (Denlinger 1991). *Leptinotarsa decemlineata* is a freeze-intolerant (Hiiesaar et al. 2001), overwintering species (Baker and Porter 2008). Adults of this species undergo diapause but juvenile hormone is not involved in its diapause induction (Hiiesaar et al. 2001). *Tribolium castaneum* has no diapause, and is also a freeze-intolerant species (Feng and He 2002), in which the processes that trigger cold tolerance cannot be expected to happen.

Further studies are needed to determine if methoprene can be used in conjunction with heat treatments to control stored-product insects. Heat treatments are currently used in two ways, either to control insects in grain or to control insects in structures (Beckett et al. 2007). Heat treatments are regularly used commercially for controlling *Tribolium* spp. in flour mills and other cereal processing facilities (Fields and White 2002; Beckett et al. 2007). Heating grain to control insects has been demonstrated at small and pilot scales, but is not used commercially. In Australia, *R. dominica* and *S. oryzae* in grain have been controlled using heat (Evans 1981). I used grain to expose *T. castaneum* to methoprene. In the heat tolerance experiment with adults, the effects were seen within the current US label rate. Additional experiments are needed to see if methoprene makes other species more sensitive to heat, making heat treatment of grain a more economically viable control option. Combining other treatments with heat and methoprene may also be tested. Further studies are required to determine if methoprene applied at label rates to floors and walls is

sufficient to render *Tribolium* adults more sensitive to heat treatments. As seen in this study and others (Mahroof et al. 2003), larvae are more heat tolerant than adults, so heat treatments are designed to control larvae. However, adults are more mobile than larvae and may escape into cool refugia within a facility and so avoid lethal temperatures. Methoprene does not degrade after 48 h at 46°C (Chapter 5). So methoprene may be a useful tool in combination with heat treatment, but further work is required to determine how it can be used at pilot scale and in an integrated pest management program to control stored-product insects.

Table 3.1. Percentage survival (mean±SEM) of *Tribolium castaneum* adults treated with methoprene and exposed to 46°C for different durations (n=4).

Methoprene concentration (ppm)	Percentage survival (mean±SEM)[a]							LT_{50} (95% CL) (h)	LT_{50} ratio (95% CL) [b]
	Duration of exposure (h)								
	0	6	9	11	13	15	17		
Water	100±0a	98.8±1.3a	61.3±6.6a	57.5±10.3a	65.0±5.0a	0±0a	0±0a	11.68 (11.03-12.30)	0.92 (0.87-0.98)
Adjuvants	100±0a	100±0a	76.3±1.3a	20.0±5.4bc	66.3±2.4a	1.3±1.3a	1.3±1.3a	10.75 (9.57-11.78)	1.0
1.67	100±0a	96.3±1.3ab	71.3±3.1a	28.8±2.4ab	30.0±5.4bc	0±0a	0±0a	10.27 (9.55-10.92)	1.05 (0.99-1.11)
3.33	100±0a	95.0±0abc	63.8±3.8a	1.3±1.3d	45.0±11.7ab	1.3±1.3a	0±0a	9.25 (8.34-10.04)	1.16 (1.09-1.24)
16.65	100±0a	82.5±6.3c	8.8±4.3c	27.5±6.6abc	5.0±2.0d	1.3±1.3a	0±0a	9.19 (8.66-9.66)	1.17 (1.11-1.24)
33.3	100±0a	91.3±2.4bc	56.3±5.5ab	7.5±4.3cd	15.0±5.4cd	0±0a	0±0a	9.06 (8.33-9.68)	1.19 (1.12-1.26)
66.6	100±0a	96.3±1.3ab	30.0±7.9b	21.3±4.3bc	7.5±2.5cd	0±0a	0±0a	9.38 (8.96-9.75)	1.15 (1.08-1.21)

[a] For a given exposure, means followed by the same letter in a column are not significantly different at $P = 0.05$ according to Tukey's test following ANOVA.

[b] LT_{50} ratio = LT_{50} for adjuvants/ LT_{50} for a particular treatment. The LT_{50} values are not significantly different at $P = 0.05$, if the 95% confidence intervals for the ratio include 1.0.

Table 3.2. Percentage adult emergence (mean±SEM) of *Tribolium castaneum* larvae treated with methoprene and exposed to 46°C for different durations (n=4).

Methoprene concentration (ppm)	Percentage emergence (mean±SEM)[a]							LT_{50} (95% CL) (h)	LT_{50} ratio (95% CL)[b]
	Duration of exposure (h)								
	0	3	6	9	12	15	18		
Water	98.8±1.3a	96.3±1.3ab	97.5±1.4a	93.8±2.4ab	91.3±2.4a	83.8±3.1ab	52.5±18.8ab	-	-
Adjuvants	100±0a	97.5±1.4ab	95.5±2.5a	97.5±1.4ab	85.0±6.1ab	87.5±6.3a	51.3±14.0ab	25.23 (17.81-21.95)	1.0
0.003	100±0a	100±0a	98.8±1.3a	98.8±1.3a	90.0±7.1a	36.3±16.5bc	80.0±4.1a	19.67 (15.91-46.68)	1.28 (0.98-1.69)
0.00825	95.0±2.9a	88.8±4.7b	95.0±2.9a	83.8±2.4bc	68.8±7.2ab	73.8±4.3ab	67.5±4.4a	29.3	0.86 (0.48-1.54)
0.0165	48.8±8.5b	55.0±4.1c	61.3±5.9b	72.5±7.8c	60.0±4.6b	47.5±11.1ab	65.0±7.4ab	-	-
0.033	16.3±5.5c	11.3±6.6d	7.5±3.2c	16.3±5.5d	11.3±6.3c	0±0d	17.5±7.2bc	-	-
0.066	3.8±2.4c	2.5±1.4d	0±0c	3.8±2.4d	2.5±1.4c	2.5±1.4cd	5.0±2.0c	-	-

[a] Means followed by the same letter in a column are not significantly different at $P= 0.05$ according to Tukey's test following ANOVA.

[b] LT_{50} ratio= LT_{50} for adjuvants/ LT_{50} for a particular treatment. The LT_{50} values are not significantly different at $P= 0.05$, if the 95% confidence intervals for the ratio include 1.0, LT_{50} could not be calculated for all methoprene concentrations.

Table 3.3. Percentage survival (mean±SEM) of *Tribolium castaneum* adults treated with methoprene and exposed to 0°C for different durations (n=4).

Cold acclimation	Methoprene concentration (ppm)	Percentage survival (mean±SEM)[a] Duration of exposure (d)								LT_{50} (95% CL) (d)	LT_{50} ratio (95% CL) [b]
		0	3	4	5	6	7	8	9		
Unacclimated	Water	78.8±2.4a	65.0±5.0a	20.0±3.5ab	3.8±2.4a	0±0a	0±0a			3.59 (3.29-3.77)	1.09 (1.02-1.19)
	Adjuvants	88.8±5.2a	83.8±1.3a	36.3±8.8ab	10.0±5.4a	1.3±1.3a	0±0a			3.89 (3.62-4.11)	1.0
	1.67	81.3±3.8a	71.3±5.5a	47.5±4.3a	2.5±1.4a	0±0a	0±0a			4.13 (3.95-4.28)	0.94 (0.88-1.01)
	3.33	93.8±1.3a	86.3±5.2a	28.8±6.9ab	2.5±1.4a	1.3±1.3a	0±0a			3.71 (3.44-3.93)	1.05 (0.98-1.12)
	16.65	81.3±13.9a	70.0±7.4a	17.5±4.8b	0±0a	0±0a	0±0a			3.56 (3.25-3.75)	1.09 (1.01-1.19)
	33.3	51.3±16.4a	80.0±6.1a	33.8±3.1ab	7.5±4.3a	0±0a	0±0a			4.12 (3.77-4.39)	0.95 (0.88-1.02)
	66.6	71.3±11.4a	87.5±4.8a	28.8±6.6ab	2.5±2.5a	0±0a	0±0a			3.88 (3.64-4.07)	1.00 (0.94-1.07)
Acclimated	Water	76.3±5.9ab	82.5±4.8a	77.5±3.2a	55.0±15.8a	40.0±3.5a	30.0±4.6b	7.5±2.5b	0±0b	6.02 (5.39-6.46)	1.19 (1.09-1.28)
	Adjuvants	75.0±6.1ab	73.8±3.1a	73.8±5.2a	75.0±5.0a	56.3±3.8a	48.8±2.4ab	13.8±3.8ab	10.0±2.9ab	7.15 (6.74-7.45)	1.0
	1.67	92.5±2.5ab	80.0±4.6a	68.8±5.2a	55.0±6.1a	53.8±2.4a	43.8±5.9ab	25.0±5.4ab	7.5±3.2ab	5.95 (5.14-6.57)	1.20 (1.08-1.34)
	3.33	67.5±9.7b	55.0±7.1a	71.3±11.6a	77.5±7.8a	71.3±6.3a	61.3±5.9a	32.5±2.5a	12.5±4.8a	8.02 (7.43-8.42)	0.89 (0.84-0.95)
	16.65	90.0±2.9ab	70.0±16.7a	66.6±13.9a	77.5±4.8a	57.5±3.2a	43.8±4.3ab	22.5±5.2ab	7.5±4.3ab	7.14 (6.03-7.76)	1.00 (0.93-1.07)
	33.3	96.3±2.4a	71.3±13.9a	63.8±9.7a	70.0±2.9a	60.0±8.7a	35.0±4.6b	16.3±5.5ab	10.0±4.6ab	5.65 (3.92-6.68)	1.27 (1.13-1.42)
	66.6	87.5±4.8ab	80.0±10.2a	90.0±4.6a	72.5±8.8a	67.5±15.9a	52.5±6.3ab	25.0±3.5ab	3.8±1.3ab	7.19 (5.69-7.69)	0.99 (0.93-1.06)

[a] For a given acclimation, means followed by the same letter in a column are not significantly different at $P = 0.05$ according to Tukey's test following ANOVA.

[b] LT_{50} ratio= LT_{50} for adjuvants/ LT_{50} for a particular treatment. The LT_{50} values are not significantly different at $P = 0.05$, if the 95% confidence intervals for the ratio include 1.0.

Table 3.4. Percentage adult emergence (mean±SEM) from *Tribolium castaneum* larvae treated with methoprene and exposed to 0°C for different durations (n=4).

Cold acclimation		Percentage survival (mean±SEM)[a]							LT_{50} (95% CL) (d)	LT_{50} ratio (95% CL)[b]
		Duration of exposure (d)								
		0	2	3	4	5	6	8		
Unacclimated	Water	92.5±5.9a	65.0±2.0ab	51.3±8.9a	21.3±6.3a	6.3±3.1a	0±0a		2.85 (2.39-3.20)	1.15 (1.01-1.31)
	Adjuvants	86.3±8.9a	82.5±7.5a	51.3±6.6a	23.8±5.2a	5.0±2.0a	0±0a		3.28 (2.92-3.55)	1.0
	0.003	95.0±2.0a	75.0±3.5ab	32.5±2.5ab	21.3±5.2a	7.5±3.2a	0±0a		2.72 (2.48-2.93)	1.21 (1.08-1.36)
	0.00825	73.8±2.4a	56.3±6.6b	36.3±4.3ab	20.0±3.5a	3.8±1.3a	0±0a		3.01 (2.39-3.39)	1.09 (0.92-1.30)
	0.0165	31.3±3.1b	17.5±2.5c	21.3±3.1b	13.8±3.8ab	0±0a	0±0a			
	0.033	1.3±1.3c	1.3±1.3d	1.3±1.3c	2.5±1.4b	1.3±1.3a	0±0a			
Acclimated	Water	87.5±3.2a	78.8±3.1a	56.3±8.5ab	53.8±6.9a	40.0±8.4a	31.3±5.2ab	8.8±2.4ab	4.42 (3.61-5.09)	1.05 (0.88-1.27)
	Adjuvants	93.8±6.3a	72.5±5.2a	70.0±7.9a	55.0±10.2a	50.0±4.1a	38.8±4.3a	12.5±6.6ab	4.66 (3.63-5.58)	1.0
	0.003	86.3±7.2a	66.3±2.4ab	57.5±5.9ab	47.5±6.6a	36.3±1.3ab	25.0±6.5ab	22.5±7.8a	4.18 (3.34-4.99)	1.11 (0.89-1.39)
	0.00825	75.0±4.6a	56.3±3.1b	37.5±4.3bc	27.5±1.4ab	27.5±7.8ab	13.8±7.7bc	5.0±2.9ab	3.25 (2.34-3.95)	1.43 (1.13-1.81)
	0.0165	27.5±7.5b	27.5±4.3c	16.3±5.5c	13.8±4.3bc	13.8±5.5bc	1.3±1.3c	1.3±1.3b	-	-
	0.033	5.0±5.0c	0±0d	1.3±1.3d	3.8±3.8b	0±0c	0±0c	0±0b	-	-

[a] For a given acclimation, means followed by the same letter in a column are not significantly different at P= 0.05 according to Tukey's test following ANOVA.

[b] LT_{50} ratio= LT_{50} for adjuvants/ LT_{50} for a particular treatment. The LT_{50} values are not significantly different at P= 0.05, if the 95% confidence intervals for the ratio include 1.0, LT_{50} could not be calculated for all methoprene concentrations.

Chapter 4. Effect of methoprene on the progeny production of *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Abstract

Tribolium castaneum (red flour beetle) is a serious insect pest of stored products around the world. It has a high reproductive rate and the current control measures for this species have several limitations including the loss of registration of insecticides, insecticide resistance and consumer concerns about chemical residues in food. The objective of this study was to determine if the juvenile hormone analogue methoprene affects progeny production of *T. castaneum*. Late-instar larvae or young adults were exposed to methoprene-treated wheat, and progeny production on fresh wheat was determined. The larvae were exposed to methoprene (0.001, 0.0165 or 0.033 ppm) or adjuvants (0.033 ppm). The adults were exposed to methoprene at 1.67, 16.65, or 66.6 ppm or adjuvants (66.6 ppm). The pairing of male and female adults was performed as untreated x untreated, treated x untreated or treated x treated, to study sex-based effects. The pairing was done in such a way to study the effects of different concentrations. When late-instar larvae had been exposed to methoprene-treated wheat kernels, either they

failed to emerge as adults, or they emerged but when paired produced almost no offspring or they emerged and offspring production was similar to that of controls. Male larvae were more susceptible to methoprene than female larvae in terms of progeny production. There was no effect on progeny production between the concentrations tested. Contrary to the results with larvae, the young adults exposed to methoprene (1.67 to 66.6 ppm) showed no reduction in offspring production. Methoprene concentrations decline with time following its application, but this research indicates that these lower concentrations of methoprene can still reduce populations of *T. castaneum* by affecting progeny production, even if adults emerge.

Keywords: methoprene, larvae, progeny, all-or-nothing, bimodal

Introduction

Synthetic insecticides, both as contact insecticides and fumigants, have been used extensively to control stored-product insects since the 1960s (Snelson 1987; Arthur 1996; Emekci 2010). However, due to many concerns with the use of currently available insecticides (Hu et al. 1999; Calumpang et al. 2001; Baker and Weaver 1993; Byrne et al. 1994), there is currently an emphasis on the use of insecticides that have bio-rational properties (Phillips and Throne 2010). This concept also includes the use of reduced-risk insecticides, such as insect growth regulators (IGRs) (Oberlander et al. 2000; Arthur 2007). There are three types of IGRs: juvenile hormone agonists, ecdysteroid agonists and chitin synthesis inhibitors (Oberlander et al. 1997).

Methoprene (Wilson and Turner 1992), hydroprene (Amos et al. 1978), fenoxycarb (Thind and Edwards 1986), and pyriproxyfen (Ishaaya et al. 1994; Oberlander et al. 1997) are JHAs. These JHAs have been used commercially to control insect pests (Oberlander and Silhacek 2000). JHAs are lethal when applied to embryos during blastokinesis (Matolin 1970; Retnakaran 1980), or to larvae, which then produce malformed pupae (Retnakaran et al. 1985; Oberlander and Silhacek 2000). Much of the research on JHAs has focused on mortality due to the disruption of development during metamorphosis (McGregor and Kramer 1975; Loschiavo 1976; Manzelli 1982; Samson et al. 1990; Moon et al. 1993; Shaaya 1993; Nayar et al. 2002).

However the effects of JH and JHAs can also be sub-lethal, as JH is involved in many physiological systems (Silhacek and Oberlander 1975; Staal 1975; Chippendale 1977; Sieber and Benz 1978; Bridges 1982; Rojas et al. 1987; Wyatt and Davey 1996; Dean and Meola 1997; Schmidt Capella and Hartfelder 1998; Teal et al. 2000; Scott 2006; Shelby et al. 2007). For example, JHAs affect diapause status (Brown 1985; Evenden et al. 2007), pheromone production (Kim et al. 2005), mating (Segura et al. 2009), heat tolerance (Chapter 3) and behavior (Robinson 1985). Juvenoids often affect reproduction. JHAs applied at the immature stages reduce offspring production during their adult stage. This is seen in the adult stage of females in both stored-product insects (Oberlander et al. 1975; Chanbang et al. 2008) and non stored-product insects (Richmond 1972; Ringo and Pratt 1978; Schmid et al. 1978; Brown and Brown 1982; Koeppe et al. 1985). Similarly in males, the presence or absence of JH during immature stages (Blaine and Dixon 1970; Koeppe et al. 1985) or exposure of immature stages to JHAs (Ferenz 1963; Dumser and Davey 1974) affects spermatogenesis during the adult stage; the effect

differs with the species. Juvenile hormone or its analogues applied to female adults can affect reproduction by affecting vitellogenesis, growth of follicles or protein synthesis in ovaries (Koeppe et al. 1985), or by causing abnormalities in ovaries (Metawally and Landa 1972). In some species, JHAs applied to adult males increase the release of pheromones, exhibit early sexual signaling such as calling, and also engage in early mating, compared to the untreated adults (Teal et al. 2000). In some other species, methoprene applied on adult males increases their sexual competitiveness (Segura et al. 2009).

The red flour beetle, *T. castaneum*, is a serious insect pest of raw grains and processed grain products (Sokoloff 1972; Li and Arbogast 1991) and is found in different habitats during post-harvest storage and processing including warehouses (Mullen 1992), flour mills (Zettler 1991), elevators (Smith and Loschiavo 1978), food processing plants (Campbell et al. 2002) and retail stores (Arbogast et al. 2000). Current control measures for *T. castaneum* include the use of contact insecticides (Arthur and Dowdy 2003), diatomaceous earth (Dowdy 1999b), fumigants such as phosphine (Rajendran 2000) or sulfuryl fluoride (Reichmuth et al. 2003), and application of low temperatures (Fields 1992) or high temperatures (Arthur 2006; Beckett et al. 2007). *Tribolium castaneum* populations have exhibited resistance to neurotoxic insecticides (Zettler 1991; Pimentel et al. 2006), some of which are still in use (Pimentel et al. 2006; Ataur et al. 2007).

Grain storage managers are moving to alternative and safer control methods in response to concerns about neurotoxic insecticides. Methoprene is one of the most-widely used JHAs (Oberlander et al. 1997; Chanbang et al. 2008), and is a reduced-risk insecticide (Arthur 2007). Although there is information on the toxicity of methoprene to

T. castaneum (Shanthy et al. 1995), little is known about how the JHAs affect the male and female reproductive systems and the reproductive capacity of this species. The objective of this study was to determine if methoprene affects the progeny production by *T. castaneum* when applied to larvae or adults.

Materials and methods

Methoprene

The commercial product Diacon II (288 g of S-methoprene/liter) (Central Life Sciences, Schaumburg, Illinois, U.S.A.) was used as the source of methoprene. Wheat was treated with a series of concentrations of methoprene in distilled water (w/w based on the active ingredient of the commercial product). Larvae were exposed to methoprene concentrations 0.001, 0.0165 or 0.033 ppm on wheat. In previous experiments, these concentrations caused approximately 25, 50 and 75% mortality, respectively (Chapter 3). The survivors at the adult stage were used to determine the effects on their progeny production. Adults were exposed to methoprene concentrations 1.67, 16.65 or 66.6 ppm on wheat. For controls, a formulation that contained all the adjuvants but no methoprene, provided by manufacturers of Diacon II, was used at equivalent highest active ingredient concentrations (0.033 ppm for larvae and 66.6 ppm for adults). In earlier experiments, the response of *T. castaneum* to these concentrations of adjuvants was not different from the response to water (Chapter 3). Hence, only the adjuvant mixture was used as a control in this experiment. All the solutions were prepared immediately before spraying.

Spraying and exposure of insects to methoprene

Adjuvant and methoprene solutions were sprayed onto hard red spring wheat, *T. aestivum*, medium containing 80% whole wheat and 20% cracked wheat (wheat medium) (moisture content 14.1%-14.5%). This wheat medium was laid on to a single grain thickness on a wax sheet. Each spray treatment (adjuvants or methoprene) was of 3 mL which was sprayed on to 300 g of the wheat medium. Spraying was carried out under a fume hood using an artist's brush (Model H#1L, Paasche Airbrush Company, Chicago, U.S.A.). Spraying on wheat was carried out in the same manner as described in Chapter 3.

Tribolium castaneum from a colony that had been in the laboratory since 1989 was used in the experiments. Insects were reared at 30°C, 60% r.h., in the dark, on a medium containing 95% unbleached white wheat flour and 5% brewer's yeast (ICN Biomedicals, Inc., Aurora, Ohio, U.S.A.) (flour medium). Insects for the experiments were produced by introducing two hundred adults to 250 g of the above medium to lay eggs for 24 h. The experiments were conducted under the same environmental conditions as used for the rearing of insects. Late-instar larvae aged 14 days were separated from the rearing medium using a sieve (425 μm mesh). This age determination was based on allowing 3 days for incubation as previously observed under the temperature, humidity and darkness used in this experiment. In the larval-exposure experiment, the larvae were introduced into the wheat medium treated with a particular concentration of methoprene (300 larvae/ 300 g medium). Between 4 and 8 days following the introduction, the pupae were separated from the treated wheat medium using a sieve (2 mm mesh) and sexed (Anonymous a 2011). Each pupa was held in a separate vial with flour medium

(approximately 1.5 g), until adult emergence. Two weeks after adult emergence, one male and one female were introduced to a vial containing flour medium (7 g). After 7 days, adults were sieved out (850 µm mesh); the flour medium containing offspring was held for 5 weeks and the progeny adults counted.

In the adult-exposure experiment, untreated pupae were separated from the rearing wheat-flour medium using a sieve (600 µm mesh), sexed, and each pupa was held in an individual vial containing flour medium (approximately 1.5 g), until adult emergence. When the adults were 2-4 days old, each adult was placed in individual vial containing wheat medium (4 g of 80% whole wheat and 20% cracked wheat) treated with a given concentration of methoprene and held for 7 days. Following this, the adults were sifted from that medium using a sieve (2 mm mesh), paired in 7 g of flour medium (each pair in a separate vial) and held for 7 days. These adults were sifted out (using 600 µm mesh), and the offspring production in the vials was assessed 5 weeks later, as described above. In both larval and adult exposure experiments, only the vials in which both adults were live at the time of sifting out from the flour medium were included in the experiment. To avoid contamination, all the procedures with spraying, introducing larvae, sexing pupae and handling adults were performed from the lowest concentration to the highest. During spraying, the new concentration was passed through the artist's brush, before spraying on to wheat. In both larval- and adult-exposure experiments, each pair of adults sifted out of the flour medium after the oviposition period were frozen at –10°C, for verification of sex in the cases where no progeny was produced.

Pairing

One adult female, either emerged from a treated larva or treated as adult, was paired with one adult male. There were four pairings: both female and male untreated, only female treated, only male treated, or both female and male treated. When both sexes were treated, the same concentration of methoprene was used for both sexes.

Statistical analysis

This experiment had two stages. The first stage was a completely randomized design (CRD) with one of four methoprene concentrations (0, 0.001, 0.0165 or 0.033 ppm) applied to larvae. The count of adults emerging was analyzed using a log-linear model (Steel et al. 1997; Mead et al. 2003; Genmod, SAS Institute, 2002-2008). Effect of concentration on adult emergence was compared using a chi-square test and were considered significantly different using a Type 1 error of 0.05 (Table 4.1).

The second stage of the experiment examined the production of progeny from the parents in the factorial set of treatments. The developing larvae were sexed as pupae and then, as adults were combined in a CRD, in a factorial experiment with 2 factors. The two factors were the methoprene concentration applied to male larvae (0, 0.001, 0.0165 or 0.033 ppm) and the same methoprene concentration applied to the females as larvae. Of 16 possible combinations, only 10 were used. One restriction was that the same concentration was used for males and females (eg. males treated with 0.001 ppm were paired only with females treated with 0.001 ppm). In the larval-exposure experiment, the offspring production was bimodal (Figure 4.1). The progeny size 30 was an approximate demarcation for the two sub sets of progeny produced by a pair of parent adults.

Therefore, I analyzed the frequency of pairs with equal or less than 30 offspring (low-progeny production) using a log-linear model (Steel et al. 1997; Mead et al. 2003; Genmod, SAS Institute, 2002-2008) and employed specific contrasts to determine differences between treatments (Type I error=0.05). As low-progeny production when both males and females were treated with adjuvants was zero, 1+X was used in the analysis of all the data. For pairs with progeny production greater than 30, data were analyzed by ANOVA procedures of SAS to determine whether they differed from the untreated control (Table 4.2).

The adult-exposure experiment was also a CRD and used a 2-factor factorial set of treatments (incomplete), as in the experiment with larvae. However, here the distribution of progeny was unimodal (Figure 4.2). Therefore, the raw counts (X) were transformed to log (1+X) scale (Zar 1999) and were analyzed using ANOVA (SAS Institute, 2002-2008). The differences from the control were tested using Dunnett's test (P=0.05) (Table 4.3).

Results

Treated as larvae

There was an increase in mortality (failure to emerge as adult) with increased methoprene concentration. As the number of live adults emerged at 0.033 ppm was not enough for pairing (Table 4.1) that concentration was not used. When both the sexes were treated with the adjuvants (control) as larvae, there was 81.9±2.4 offspring/ pair (mean±SEM), the frequency distribution of progeny production was normal (Kolmogorov-Smirnov test; P=0.122) and unimodal (Fig. 4.1A). When one or both of the

sexes was treated with methoprene, this distribution was not normal (Kolmogorov-Smirnov; $P<0.010$). Also, it was bimodal (Fig. 4.1 B-G), with two distinct responses to methoprene; either a pair had very low-progeny production (most pairs had zero progeny), or a pair's progeny production was similar to that for controls.

The frequency of pairs with low-progeny production (equal or less than 30 offspring) was calculated (Table 4.2). Compared to the progeny group size produced by males and females both untreated at the larval stage, the frequencies of production of the low-progeny-production group size significantly higher when either sex or both sexes were treated with methoprene concentrations 0.001 or 0.0165 ppm (LR χ^2= 55.10 with 6 d.f., $P<0.0001$). In general, males were significantly more affected than females (LR χ^2= 14.0 with 3 d.f., P=0.0029). However, at individual concentration, this was significant at 0.0165 ppm (LR χ^2= 10.34 with 1 d.f., P=0.0013) but there was no difference at 0.001 ppm (LR χ^2= 3.65 with 1 d.f., P=0.0559). Concentration did not affect the frequency of low-progeny production, with only females treated (LR χ^2= 0.51 with 1 d.f., P=0.4749), with only males treated (LR χ^2= 0.33 with 1 d.f., P=0.5684) or both sexes treated (LR χ^2= 0.69 with 1 d.f., P=0.4078). Furthermore, means of the progeny sub-groups with greater than 30 offspring in any of the treatments did not significantly differ from untreated controls (ANOVA, $F_{6,95}$=1.34, P=0.2480, Table 4.2).

When both sexes were exposed to methoprene, the frequency of pairs with low-progeny production was higher than for the female-only treatment either at 0.001 ppm (LR χ^2= 10.24 with 1 d.f., P=0.0014) or at 0.0165 ppm (LR χ^2= 10.16 with 1 d.f., P=0.0014). This was significant even when the data sets for the two concentrations were combined (LR χ^2= 20.41 with 1 d.f., $P<0.0001$). In contrast, low-progeny production with

male-only treatment was not significantly different from that when both sexes were treated either at 0.001 ppm (LR χ^2= 1.73 with 1 d.f., P=0.1880), at 0.0165 ppm (LR χ^2= 0.0 with 1 d.f., P=0.9454), or in the combined data set (LR χ^2= 0.79 with 1 d.f., P=0.3746) (Table 4.2). The effect of methoprene on the probability of pairs having low-progeny production when both sexes of the pair were treated can be calculated by considering individual probability levels of at least one individual having low-progeny production. It is assumed that having at least one adult of the pair with low-progeny production will cause the pair to have low-progeny production, as none of the untreated pairs had the low-progeny production. If a female was treated with 0.001 ppm methoprene, then the probability it would have low-progeny production would be 0.292, for males it would be 0.560. Based on these individual probabilities, a predicted value for the expected probability can be calculated as, (f x m) + (F x m) + (f x M) where f, m, F and M represent probabilities of having a low-progeny-production female, a low-progeny-production male, a normal female and a normal male, respectively. Accordingly, for 0.001 ppm methoprene, the predicted value for the probability of low-progeny production is 0.689 obtained as (0.292 x 0.560) + (0.780 x 0.560) + (0.292 x 0.440), which is similar to 0.733, the actual probability obtained in the experiment. For 0.0165 ppm methoprene, the predicted probability obtained from a calculation as discussed above is 0.647, which is similar to the observed value of 0.633.

Treated as adults

When both the sexes were treated with the adjuvants (control) as adults, the frequency distribution of progeny production was unimodal, with 88.5±3.4 offspring/ pair (mean±SEM) (Figure 4.2 A). Unlike the larvae, the frequency distribution of progeny production of treated adults was unimodal (Fig. 4.2 B). In general, methoprene did not reduce progeny production when young adults were exposed to methoprene (Table 4.3). The only exception was the progeny production of pairs in which males were treated with 16.65 ppm, which was lower than when both the sexes were untreated.

Discussion

There were three outcomes to methoprene treatment of larvae in this study: 1. larvae failed to emerge as adults; 2. larvae emerged as adults, but produced almost no offspring; or 3. larvae emerged as adults and produced similar numbers of offspring to those from controls. Failure to emerge as an adult due to methoprene treatment is well documented (Retnakaran et al. 1985; Oberlander and Silhacek 2000; Chapter 3). In insects with complete metamorphosis, JH titers generally remain low in the later part of the last larval instar and the pupal stage for normal development of insects (Nation 2002). High JHA levels during immature stages disrupt development of the insect (Silhacek and Oberlander 1975) so much so, that the insect dies (Jenson et al. 2009).

In the second type of outcome, adults had normal morphology and movement but they produced very few offspring. Both male and female reproductive systems develop during the larval and pupal stages (Sokoloff 1972; Parthasarathy et al. 2010a). This study indicates that methoprene may disrupt this reproductive development in those *T.*

castaneum that withstand the lethal effects and developed into adults. Other insects also show similar effects of reduced offspring production when larvae are exposed to JHAs: *C. occidentalis* (Richmond 1972; Robertson and Kimball 1979) and *A. orana* (Schmid et al. 1978).

In this study, progeny production of both males and females were adversely affected, with males being more sensitive to methoprene than females. There are several mechanisms by which methoprene could have suppressed progeny production. Exposure of immature stages of males to JHAs disrupts spermatogenesis (Dumser and Davey 1974) and functioning of accessory glands in some insect species; the degree to which the target tissue is affected differs with the species (Koeppe et al. 1985). Supportive tissues and aedeagus might also be affected by JH (Koeppe et al. 1985). In females, JH affects the development of oviducts (Koeppe et al. 1985), follicular growth (Koeppe et al. 1980), oocyte maturation (Koeppe et al. 1985) and functioning of accessory glands (Bodenstein and Sprague 1959; Koeppe et al. 1985). Also, externally applied JHAs can affect the morphology of genitalia (DeVries and Brown 1977). Males could be more sensitive to methoprene because one or more of the tissues of the male reproductive system may be more sensitive than tissues in the female reproductive system.

An important finding in this study was that only some of the exposed larvae were susceptible to methoprene, as evidenced by the bimodal distribution of progeny produced. There are several possible explanations for this effect. Genetic differences may result in individual insects having different susceptibility to methoprene through differences in the uptake, degradation or susceptibility of target tissues (Arthur 1996; Retnakaran et al. 1985; Zettler 1991). Furthermore, individual insects may have received different doses of

methoprene because of variation in methoprene distribution in the grain sample; different rates of movement, development or feeding. Finally, it could be that there is a defined window of sensitivity during the development of *T. castaneum* larvae, during which methoprene prevents the normal development of reproductive systems. Although all larvae used in the experiment were from the eggs laid within 24 h period, there are always differences in the rate of development. Thus, larvae exposed to methoprene within the window of sensitivity would have non-functioning reproductive systems as adults. In contrast, those larvae exposed to methoprene outside the window of sensitivity would not be affected.

There are several examples that insects have a window of sensitivity to JH or JHAs. Larvae are sensitive and adults are not sensitive to JH and JHA (Oberlander and Silhacek 2000; Staal 1975; Koeppe et al. 1985). Within the egg and larval stages also, there are examples of JH-sensitive phases. Development of embryo cuticle in *Acheta domesticus* L. (Orthoptera: Gryllidae) is sensitive to pyriproxyfen only for a few days (Erezyilmaz et al. 2004). With *O. fasciatus*, JH must be present during the fourth instar larvae for the normal development of accessory glands, and the JH deficiency during that period cannot be recovered by having JH at a later stage (Koeppe et al. 1985). In *C. pomonella*, methoprene reduces fecundity when applied to two-day and six-day old fifth instar female larvae. However, the fecundity is not reduced when methoprene is applied to four-day old female larvae of this instar (Brown and Brown 1982). *Melipona scutellaris* Latreille (Hymenoptera: Apidae) has a JH-mediated control of female genes within a specific window during the late larval stage (L_3) (Vieira et al. 2006). It could be that males have a longer window of sensitivity than females, a possible explanation for

the greater effects observed in males. However, further research is required to determine the exact mechanism by which methoprene caused *T. castaneum* to become sterile.

In the experiment with *T. castaneum* 2-4 d old adults, methoprene did not alter their progeny production. Based on progeny-production data, maturation of the reproductive systems in *T. castaneum* of both sexes takes approximately five days after eclosion (Erdman 1964). The data in the current experiment suggest that juvenile hormone may not be involved in the final stages of development of the reproductive systems in *T. castaneum*, once the adult has emerged. Alternatively, juvenile hormone levels may be high in the adult (Retnakaran et al. 1985; Wyatt and Davey 1996; Parthasarathy et al. 2009; Parthasarathy et al. 2010b), and external application of methoprene may not be enough to disrupt the reproductive systems. In general, there is no adult mortality due to methoprene application (Oberlander and Silhacek 2000; Chapter 3), but methoprene or other JHAs have adversely affected the reproductive systems of adults in several other species: *T. confusum* (McGregor and Kramer 1975), *L. serricorne* (Marzke et al. 1977), *C. maculatus* (Rup and Chopra 1984) and *R. dominica* (Daglish and Pulvirenti 1998).

This study revealed that the progeny production of *T. castaneum* treated as larvae was reduced at 0.001 ppm, approximately 1600-5000 times lower than the label rate of 1-5 ppm methoprene on wheat (Anonymous b 2011). The progeny production was reduced from approximately 82 offspring/week/female in untreated wheat to 24 offspring/week/female in treated wheat. This has a number of implications for the use of methoprene in stored-grain insect pest management. Presence of adults in grain treated with methoprene may be less of a concern, as the majority of these adults may not be able

to produce progeny. Methoprene degrades with time, dropping below the concentrations that prevent adult emergence. These data show that methoprene should suppress population build-up for much longer periods of time by reducing the progeny production of the survivors. Grain should be treated at the label rate, however, there are a number of reasons that insects would not be exposed to the full label rate during a practical situation, such as incomplete coverage of grain to the sprayed concentration, movement of insects through grain mass without adequate exposure or degradation of methoprene over time. Larvae exposed to sub-lethal concentrations of methoprene may emerge, but their progeny production as adults may be adversely affected. Previous studies suggest that methoprene applied on wheat remains effective in controlling the development of insects for months (Mian and Mulla 1982a; 1982b). This study reveals that methoprene will reduce progeny production even after its dose drops below the label rate. Thus, the study contributes to expand the uses of methoprene in insect pest management.

Further work is required to determine if the effects of methoprene on progeny production are seen at concentrations lower than tested in this study, and whether similar effects are observed in other stored-product insects. Methoprene reduces the heat tolerance in adult *T. castaneum* (Chapter 3). It would be interesting to determine if larvae that survive methoprene exposure and emerge as adults have reduced heat tolerance. As described above, these findings not only provide insight into the mode of action of methoprene on reproductive physiology in insects, but also pave the way for enhancing the use of methoprene as a reduced-risk insecticide.

Table 4.1. Percentage adult emergence from *Tribolium castaneum* larvae treated with different methoprene concentrations.

Methoprene Concentration (ppm)	Emergence to adult (%)[1]	Number of pupae sexed
0	94.1a	253
0.001	62.8b	441
0.0165	27.9c	283
0.033	4.6d	787

1. Percentage emergence followed by the same letter are not significantly different (LR χ^2= 969.10 with 3 d.f., P<0.0001).

Table 4.2. Progeny production (mean±SEM) of one adult pair of *Tribolium castaneum* exposed to methoprene as larvae.

Methoprene concentration (ppm)		Progeny production (mean±SEM)		Percentage of pairs with low-progeny production	Total number of pairs
Male	Female	Production by pairs producing ≤ 30 offspring	Production by pairs producing >30 offspring		
0	0	-	81.9±2.4	0	28
0	0.001	3.4±3.4	80.1±3.4	29.2	24
0.001	0	0.1±0.1	76.3±7.7	56.0	25
0.001	0.001	1.8±1.2	79.6±5.6	73.3	30
0	0	-	81.9±2.4	0	28
0	0.0165	0.2±0.2	77.5±3.8	20.0	25
0.0165	0	3.4±2.3	85.1±7.7	64.0	25
0.0165	0.0165	1.1±0.9	67.0±5.5	63.3	30

Table 4.3. Progeny production (mean±SEM) by one adult pair of *Tribolium castaneum* exposed to methoprene as adults.

Methoprene concentration (ppm)		No. of offspring (Mean±SEM)	Number of pairs
Male	Female		
0	0	88.5±3.4	39
0	1.67	84.5±3.0	30
1.67	0	81.3±3.4	30
1.67	1.67	85.2±2.4	30
0	0	88.5±3.4	39
0	16.65	88.4±3.2	30
16.65	0	71.4±3.0[1]	30
16.65	16.65	84.7±3.8	30
0	0	88.5±3.4	39
0	66.6	98.4±3.0	30
66.6	0	84.8±4.1	40
66.6	66.6	81.7±3.6	30

1. Significantly different from control (neither female nor male treated with methoprene); Dunnett's test, P=0.05.

Figure 4.1. Frequency distribution of progeny production by pairs of parent adults, exposed to different methoprene treatments as larvae; A. both sexes untreated, B. females treated at 0.001 ppm, C. males treated at 0.001 ppm, D. both sexes treated at 0.001 ppm, E. females treated at 0.0165 ppm, F. males treated at 0.0165 ppm, G. both sexes treated at 0.0165 ppm.

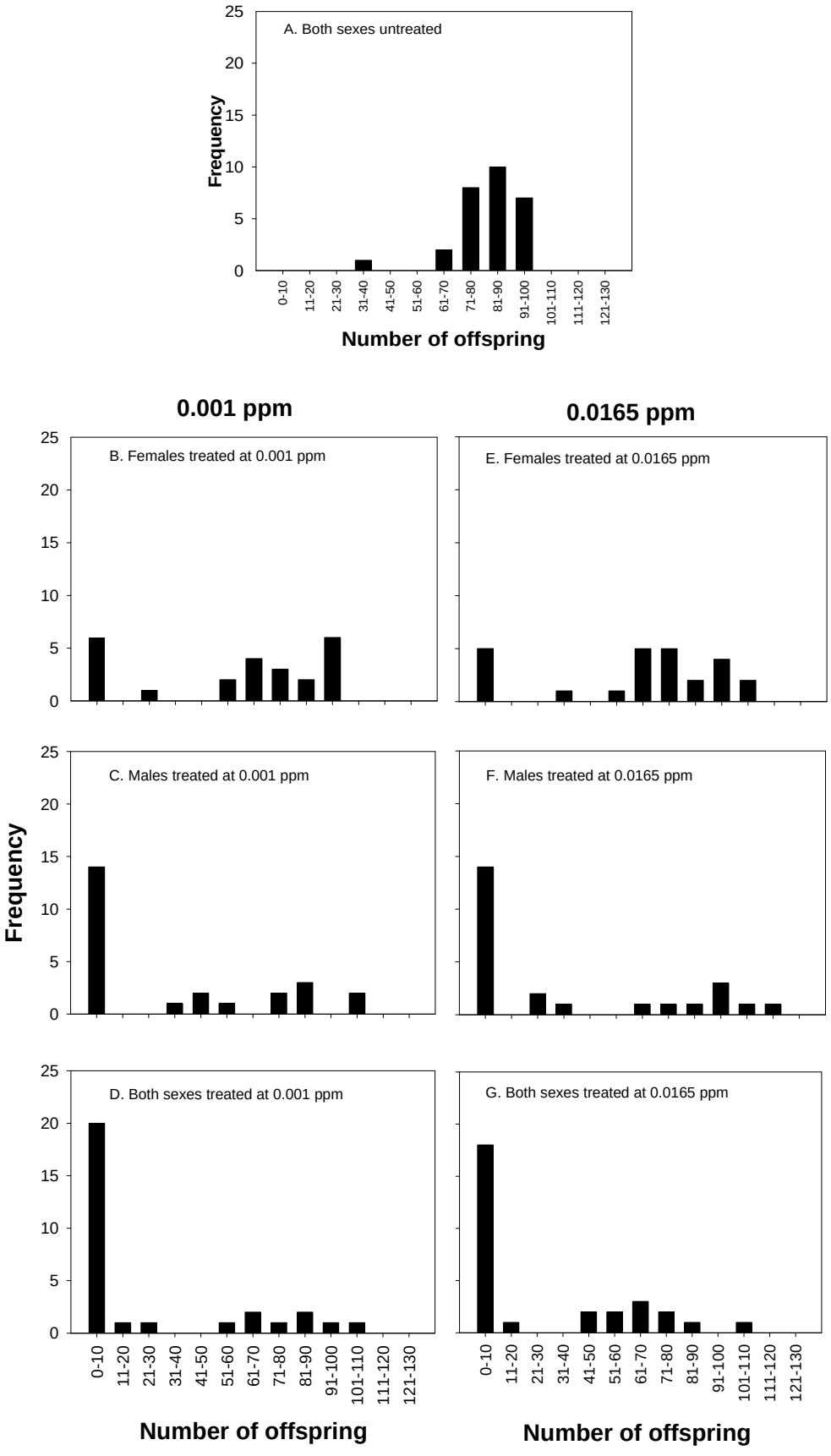
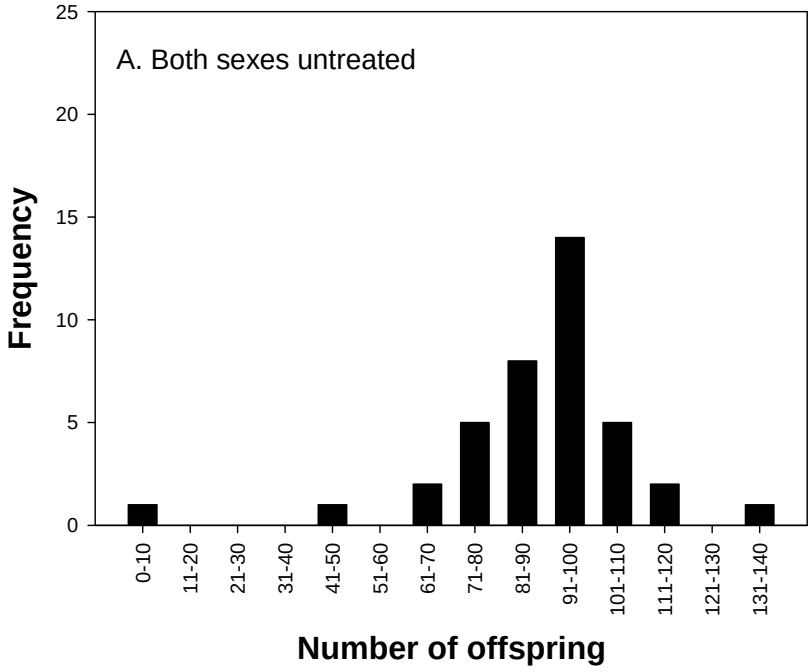
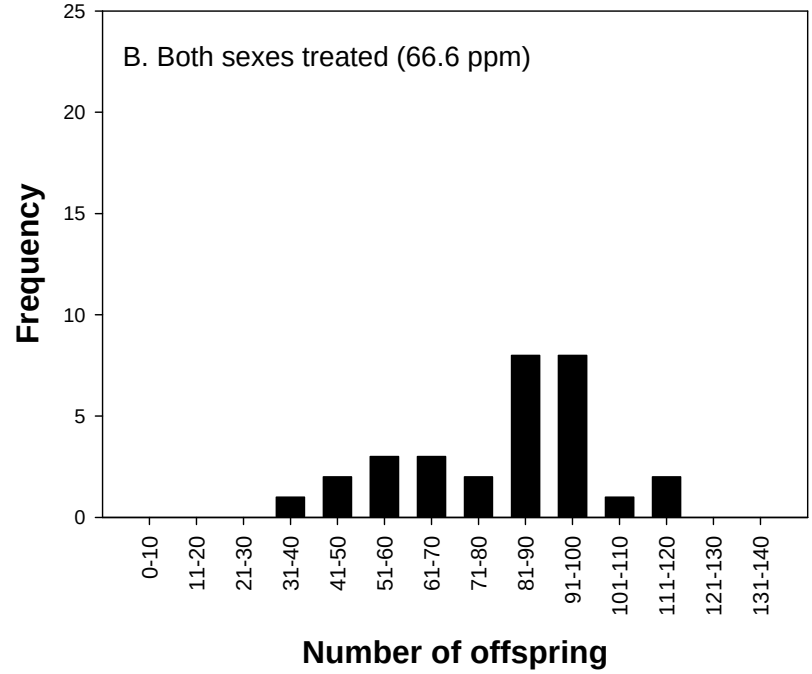

A. Both sexes untreated
Frequency
Number of offspring
0.001 ppm
0.0165 ppm
B. Females treated at 0.001 ppm
E. Females treated at 0.0165 ppm
C. Males treated at 0.001 ppm
F. Males treated at 0.0165 ppm
D. Both sexes treated at 0.001 ppm
G. Both sexes treated at 0.0165 ppm
0-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
101-110
111-120
121-130

Figure 4.2. Frequency distribution of progeny production by pairs of parent adults, exposed to different methoprene treatments as adults; A. both sexes untreated, B. both sexes treated with 66.6 ppm.





Chapter 5. Residual efficacy of methoprene on *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

Abstract

The residual efficacy of the juvenile hormone analogue, methoprene, under different storage conditions, was evaluated in bioassays using larvae of *T. castaneum*, the red flour beetle. Concrete and varnished wood were placed in Petri dishes, sprayed with the label rate of methoprene (0.0003 mg methoprene/cm^2 Diacon II) or adjuvants, subjected to different storage conditions and twenty late-instar larvae (10-12 days old since hatching) were introduced to determine the effect of treatment on adult emergence at 30ºC. Methoprene was toxic to larvae and significantly reduced the adult emergence, compared to those treated with adjuvants. When the surfaces were stored at 20, 30 or 35ºC for 0, 8, 12, 16, 20 or 24 weeks, percentage adult emergence on concrete increased with time, showing a simple linear regression. In contrast, there was no adult emergence on varnished wood for the entire 24-week storage period. Temperature did not affect methoprene efficacy. Flour (7 g) on concrete surfaces reduced efficacy of methoprene. However, this did not occur on varnished-wood surfaces. To determine the effect of cleaning on residual efficacy, the concrete surfaces either without flour or with flour (7 g) were first maintained at 30ºC for different durations, and then bioassayed. The surfaces with flour were cleaned at different frequencies; no cleaning, cleaned once in four weeks or cleaned once a week. The adult emergence on surfaces that did not contain flour was significantly lower than the average adult emergence on surfaces that received flour and cleaning. However, surfaces with different cleaning frequencies had no differences in

adult emergence. Residual efficacy of methoprene was also tested at high temperatures. Concrete and varnished wood treated with methoprene were exposed to 65ºC for 0, 6, 12, 24 or 48 h and were tested with larval bioassays. There was no decline in residual efficacy with time. Another experiment was conducted to determine the residual efficacy of methoprene on wheat. Wheat sprayed with different concentrations of methoprene (0, 0.003, 0.00825, 0.0165 or 0.033 ppm) were held at 46ºC for 0, 3, 6, 12, 24 or 48 h, and used in a larval bioassay. There was no decline in residual efficacy with time at any of the concentrations. These studies suggest that application of methoprene on varnished-wood surfaces would reduce the reapplication frequency compared to concrete surfaces. Stability of methoprene from 20-65ºC suggests the possibility of its use under different storage conditions and in commodity heat treatments.

Key words: methoprene, concrete, varnished wood, residual efficacy, temperature

Introduction

Tribolium castaneum is a common insect pest found in different types of storage facilities: flour mills (Zettler 1991), processing plants (Campbell et al. 2002), warehouses (Mullen 1992) and retail stores (Arbogast et al. 2000). Control methods for this species include the use of residual insecticides such as cyfluthrin (Arthur 1998a, 1998b; Arthur and Dowdy 2003) or diatomaceous earth (Dowdy 1999b); fumigation with phosphine (Rajendran 2000) or sulfuryl fluoride (Reichmuth et al. 2003); exposure to low temperatures (Fields 1992) or high temperatures (Arthur 2006; Beckett et al. 2007). Treating floors, walls and shelves with residual insecticides is a common method to reduce populations in grain processing facilities, warehouses and retail outlets. For example, cyfluthrin is applied on surfaces, cracks and crevices (Arthur 1998a, 1998b).

Methoprene is a JHA which is an IGR (Chanbang et al. 2008). It prevents the development of immature insects to the adult stages (Oberlander and Silhacek 2000). Methoprene has low mammalian toxicity (oral LD_{50} for rats >34,600 mg/kg) (Ware and Whitacre 2004), and is effective at very low rates (Hoppe 1981; Chapter 3). Methoprene is a registered insecticide in the U.S.A. (Jenson et al. 2009) and Australia (Daglish 2008; Daglish and Nayak 2010) to be used as a grain protectant and for crack-and-crevice treatment. In Canada, methoprene is registered for mosquito control (Health Canada 2001), but it is not yet registered for stored-product uses. Methoprene has the outstanding feature of being insect specific (Oberlander and Silhacek 2000), and thus designated a reduced-risk insecticide (Arthur 2007). Resistance to many stored-product insecticides has been a problem for over 40 years (Champ and Campbell-Brown 1970; Champ and Dyte 1976; Collins and Wilson 1987; Collins 1990; Arthur 1996; Subramanyam and

Hagstrum 1996), and methoprene has proven to be a good alternative for insecticide-resistant insect populations (Oberlander et al. 1997). Methoprene is very toxic to *T. castaneum* larvae, preventing their development to adults (Shanthy et al. 1995; Arthur 2008; Chapter 3). However, most of these studies have evaluated the effect of methoprene when applied to the food medium, and there is little information to guide its use as a surface treatment in flour mills and warehouses.

The amount of insecticide present on a surface decreases over time (Arthur 1997b; Arthur 1998b). The residual efficacy can be defined as the lethal effect of an insecticide left over time (Ware and Whitacre 2004). In stored-product protection, the storage conditions and practices may also affect the residual efficacy of an insecticide. Heat treatments are widely used in flour mills and processing plants to control *Tribolium* spp. (Fields and White 2002; Beckett et al. 2007). Heat is also used to control insects in commodities (Evans 1981). Hence, it is important to investigate the efficacy of methoprene over time, under different storage practices, including exposure to high temperature. The first objective of this study was to determine the effect of surface material, temperature, duration of exposure and cleaning on the efficacy of methoprene. The second objective was to determine the residual efficacy of methoprene applied on wheat and then held at high temperature.

Materials and methods

Experimental arenas

Plastic Petri dishes (8.4 cm in diameter) (Fisher Scientific Company, Ottawa, Ontario) were used to contain the different surfaces. The bottom portions of these Petri dishes were filled with driveway patching concrete (Rockkite®) (Hartline Products Co., Inc., Cleveland, Ohio, U.S.A.) or fir plywood varnished with Varathane (Rust-Oleum Corporation, Vernon Hills, Illinois, U.S.A.). One kilogram concrete powder was mixed with 500 mL distilled water for approximately 5 min using a magnetic stirrer. This was poured into the bottom portions of Petri dishes and allowed to dry. Varnished-wood disks were placed inside the bottom portions of Petri dishes, and were caulked (Draftstop, Canadian Adhesives Ltd., Brampton, Canada) to keep insects on the top surface. A few weeks after preparation, the Petri dishes were used in the experiment.

Spraying of methoprene

Diacon II (Central Life Sciences, Schaumburg, Illinois, U.S.A.) was used as the source of methoprene. A solution provided by the manufacturers that had all the adjuvants, but no methoprene, was used as the control. The solutions were diluted using distilled water. In the surface-treatment experiments, Diacon II was applied at the label rate (0.0003 mg methoprene/ cm^2). In the experiment which examined the residual efficacy of methoprene on wheat, a series of methoprene concentrations was used. For different replicates, independent solutions were prepared. The adjuvant solution used for spraying was prepared by diluting a volume of adjuvants equivalent to that used with Diacon II. Solutions were prepared immediately before spraying. These solutions were

applied in a fume hood using an artist's air brush (Model H#1L, Paasche Airbrush Company, Chicago, U.S.A.), at 103 kPa. For a given experiment, during each replicate spraying, the order of spraying of surfaces was randomized. To avoid contamination, spraying with adjuvants was done first, and those surfaces sprayed with adjuvants were kept in a separate laboratory. After the spraying, the surfaces were air dried overnight.

Insect bioassay

Tribolium castaneum late-instar larvae (10-12 days old from hatching) reared on flour medium contained 95% unbleached whole wheat flour + 5% brewer's yeast (MP Biomedicals, LLC, Solon, Ohio, U.S.A.) were used as test insects. These larval cultures were prepared by introducing 200 adults to 250 g of the flour medium for 3 d for oviposition in the incubators maintained at 30.2, 29.6-30.5ºC; 42.1, 36.8-58.5% r.h. (mean, range) (HOBO data loggers, Onset Computer Corporation, MA, U.S.A) and complete darkness. During the bioassay, 0.50±0.01 g of the flour medium measured using an analytical balance (Mettler AE 166, Greifensee, Zurich, Switzerland) was placed in a small pile on the test surface to facilitate adult emergence (Arthur 2001). Twenty *T. castaneum* late-instar larvae of approximately equal size were placed on Petri dishes and held at 30±1ºC with no light. Adult emergence was assessed at 2 and 3 weeks following the introduction of larvae. Adults present after two weeks were removed to minimize cannibalism.

Effect of surface, temperature and duration

Methoprene or adjuvant mixture was sprayed on concrete and varnished-wood surfaces as described above. Following spraying, the surfaces were air dried overnight and these surfaces were then maintained at 19.7, 19.4-20.1; 29.9, 29.5-30.0; or 34.6, 34.4-34.7ºC (mean, range) for 0, 8, 12, 16, 20 or 24 weeks before used in the larval bioassay as described above to determine the efficacy of methoprene at preventing emergence to adults. In the bioassay conducted after a particular duration, four replicates from each surface treated either with adjuvants or methoprene and held at a particular temperature were tested.

Effect of flour and cleaning

To test the effect of flour on the residual efficacy of methoprene at 30ºC, a set of concrete and varnished-wood surfaces treated with adjuvants or methoprene were provided with 7 g of unbleached wheat flour to cover the surfaces (about 0.5-0.75 cm in depth) and were held at 30ºC for 0, 8, 12, 16, 20 or 24 weeks. At the end of each duration, the wheat flour was removed (the flour poured out and the remaining flour brushed off with a cosmetic brush (K4750, Symak Sales Co. Inc., Montreal, Canada)), and a larval bioassay conducted as previously described.

To test for the effect of cleaning on the residual efficacy of methoprene, concrete surfaces sprayed with adjuvants or methoprene were held at 30ºC, and subjected to four cleaning protocols. These were flour (7 g) and no cleaning, flour and cleaning every 4 weeks, flour and cleaning once a week, and no flour and no cleaning. Each cleaning was followed by the provision of another 7 g of flour on the surfaces. Cleaning entailed

dumping of flour and brushing off remaining flour with the cosmetic brush. The larval bioassay was conducted at 30ºC, at the end of 0, 4, 8 or 16 weeks. When the cleaning and bioassays were coincided every 4 weeks, cleaning was performed immediately before the bioassay. For the bioassay at 0 weeks, cleaning was represented by the addition of flour (7 g) to surfaces and cleaning it after 24 h. There were four replicates per cleaning protocol.

Effect of high temperatures on residual efficacy

Concrete and varnished-wood surfaces were sprayed, as previously described, with adjuvants or methoprene, and were air dried overnight. These surfaces were held at 65.1-65.9ºC for 0, 6, 12, 24 or 48 h in an oven (Thermocenter TC40/ TC100, Salvis Lab, Rotkreuz, Switzerland). Each Petri dish was covered by its lid and placed directly on an oven shelf, without stacking or touching adjacent Petri dishes. After removal from the oven, dishes were allowed to cool to room temperatures and larval bioassays conducted. There were four replicates for each treatment.

Effect of humidity on bioassay

The surfaces treated with adjuvants and methoprene were kept in separate incubators to prevent possible contamination. Differences in relative humidity were detected between the two incubators holding the surfaces treated with adjuvants and methoprene. The surfaces treated with adjuvants were exposed to higher relative humidity 20.3, 15.5-27.8% (mean, range) during the bioassay than those surfaces treated with methoprene 16.3, 15.5-17.1% (mean, range). An additional bioassay was conducted

to verify that the reduced adult emergence on methoprene-treated surfaces was not due to the low relative humidity. This was designed by using the concrete and varnished-wood surfaces first treated with adjuvants or methoprene, and held at 35ºC. This additional bioassay was conducted simultaneously with the regular bioassay after 24-weeks. In this additional bioassay, the concrete and varnished-wood surfaces treated with adjuvants were placed in the low relative humidity, in which regular bioassay with methoprene-treated surfaces was conducted. Alternatively, the bioassay on surfaces treated with methoprene was conducted in high relative humidity conditions which held the surfaces treated with adjuvants in the regular bioassay.

Residual efficacy of methoprene on wheat maintained at high temperature

Hard red spring wheat *T. aestivum* (14% moisture content) medium containing 80% whole wheat and 20% cracked wheat was sprayed with different concentrations of methoprene (0.003, 0.00825, 0.0165 or 0.033 ppm). Adjuvant mixture or distilled water was sprayed as for the control. For a given concentration, there were four replicate sprayings. For each spraying, 300 g of the wheat medium was laid as a single grain layer on a wax sheet and sprayed with 3 mL of a particular concentration, using an artist's brush (Model H#1L, Paasche Airbrush Company, Chicago, U.S.A.). The method of spraying was as described in Chapter 3. The sprayed wheat was hand tumbled for 30 s (Arthur 2004) in a plastic bag. Each wheat sample (300 g) sprayed with a particular concentration, was divided into six 20 g samples. Each of these was placed into a 35 mL glass vial and was used in the six durations of heat exposure. Vials were held at 46±0.5°C for 0, 3, 6, 12, 24 or 48 h in an oven (Thermocenter TC40/ TC100, Salvis Lab, Rotkreuz,

Switzerland), then transferred to an incubator maintained at 30°C. After 24 h, twenty similarly-sized *T. castaneum* late-instar larvae were introduced in to each vial and held at 30°C in total darkness. After 3 weeks, adults were counted. For a given combination of treatment concentration and duration, four replicates were used.

Experimental design

The experiment that examined the effects of surface, temperature and duration had a complete randomized four factor-factorial design. The four factors were the surface type (concrete or varnished wood), treatment (adjuvants or methoprene), temperature to which the surfaces were exposed (20, 30 or 35ºC) and duration at each temperature prior to the bioassay (0, 8, 12, 16, 20 or 24 weeks).

The experiment on effect of flour used complete randomized four-factor factorial design. The four factors were the surface type (concrete or varnished wood), treatment (adjuvants or methoprene), presence/ absence of flour, and duration at 30ºC prior to the bioassay (0, 8, 12, 16, 20 or 24 weeks).

The experiment on effect of cleaning had a complete randomized three-factor factorial design. The three factors were the treatment (adjuvants or methoprene), presence/absence of flour and duration at 30ºC prior to the bioassay (0, 4, 8 or 16 weeks).

The experiment on the effect of high temperatures on residual efficacy used a complete randomized three-factor factorial design. The three factors were the treatment (adjuvants or methoprene), surface type (concrete or varnished wood) and duration at 65ºC (0, 6, 12, 24 or 48 h).

The experiment on the effect of humidity on bioassay had a complete randomized three-factor factorial design. The three factors were humidity level, treatment (adjuvants or methoprene) and surface type (concrete or varnished wood).

The experiment on the residual efficacy of methoprene on wheat maintained at high temperature used a complete randomized two-factor factorial design. The two factors were the concentration (water, adjuvants, 0.003, 0.00825, 0.0165 or 0.033 ppm) and duration at 46ºC (0, 3, 6, 12, 24 or 48 h).

Data analysis

Residual efficacy of methoprene was determined as the percentage of adults emerged. The percentage of adults emerged was transformed using the square root of the arcsine to accommodate the unequal variances associated with percentage data. These data were analyzed by factorial ANOVA procedures of SAS (SAS Institute, 2002-2008). Significance was tested at α=0.05 level.

The specific analysis performed varied with the experiment. In general, the data were analyzed to find out the effect of treatment (methoprene vs. adjuvants), surface type (concrete vs. varnished wood), temperature of exposure, duration of exposure, and also their interactions. Following the initial analysis, significant effects were further characterized by statistical modeling. In the experiment that tested effects of surface, temperature and duration, the emergence was regressed with the duration to determine whether there was simple-linear regression or polynomial relationship. In the experiment that tested effect of cleaning, contrasts were employed to determine the effects of flour and cleaning on the adult emergence. In the experiment that tested residual efficacy of

methoprene on wheat maintained at high temperature, contrasts were employed to determine whether there was a difference between water and adjuvants on adult emergence. Also, for each concentration, the emergence was regressed with the duration to determine the type of relationship.

Results

Effect of surface, temperature and duration

Surfaces treated with adjuvants had significantly higher adult emergence than those treated with methoprene (ANOVA, treatment, $F_{1,216}$=1978.43, P<0.0001). With adjuvants, concrete and varnished-wood surfaces had 99.9±0.1% and 99.6±0.2% adult emergence, respectively. As the adjuvants did not reduce the adult emergence, I analysed only the data with methoprene treatments. Of methoprene treated surfaces, concrete had greater adult emergence than varnished-wood surfaces (Table 5.1, ANOVA, $F_{1,108}$=111.07, P<0.0001). On varnished-wood surfaces there was no adult emergence, even after 24 weeks. As there was no variation from zero emergence on varnished wood, a separate ANOVA was performed just for concrete. On concrete surfaces, the adult emergence increased with the increased duration (ANOVA, $F_{5,54}$=5.83, P=0.0002), but there were no significant differences in the adult emergence between the three temperatures (ANOVA, $F_{2,54}$=1.92, P=0.1571). Furthermore, the interaction of temperature x duration was also not significant (ANOVA, $F_{10,54}$=0.35, P=0.9608). Therefore, for a given duration, the emergence at three temperatures was pooled. These average adult emergences were approximately 22 and 50% after 8 and 16 weeks, respectively (Table 5.1). On these pooled data, the average emergence was significantly

affected by the duration (ANOVA, $F_{5,66}$=6.27, P<0.0001). To test the hypothesis of the linearity of regression between emergence and duration, the linear effect of duration was fitted (ANOVA, $F_{1,4}$=16.49, P=0.0153) (Figure 5.1). Lack-of-fit was tested and found non-significant (ANOVA, $F_{4,66}$=1.53, P=0.204), indicating that there is no significant non-linearity in the pattern of emergence over the duration.

Effect of flour

The surfaces treated with adjuvants had at least 93.8±3.8% adult emergence. Surfaces treated with methoprene had lower adult emergence than adjuvants (ANOVA, $F_{1,144}$=1892.64, P<0.0001). For methoprene, residual efficacy was affected by surface material (ANOVA, $F_{1,72}$=295.17, P<0.0001). Concrete surfaces had higher adult emergence than varnished wood (Table 5.2). The adult emergence on the surfaces with flour was compared with that on corresponding surfaces which had similar conditions and used in the previous experiment (same surface type sprayed with the same solution), but had no flour. There was no adult emergence on varnished-wood surfaces treated with methoprene, both in the presence or absence of flour. In general, the residual efficacy changed with the presence or absence of flour (ANOVA, $F_{1,72}$=70.09, P<0.0001) and duration (ANOVA, $F_{5,72}$=10.64, P<0.0001). The interaction of surface x duration was also significant (ANOVA, $F_{5,72}$=10.64, P<0.0001). As the emergence on varnished wood did not change from zero during the 24 weeks, subsequent ANOVA was performed only on concrete. There was a significant difference in the adult emergence on concrete surfaces with respect to duration (ANOVA, $F_{5,36}$=10.64, P<0.0001), presence of flour (ANOVA, $F_{1,36}$=70.09, P<0.0001), and the interaction of duration x presence of flour

(ANOVA, $F_{5,36}$=3.05, P=0.0213). This means that the amount of change in the residual efficacy due to the presence of flour differed with the lapse of time (eg. amount of change at 24 weeks was different than that at 0 weeks).

Effect of cleaning

The concrete surfaces treated with adjuvants had significantly higher adult emergence than those treated with methoprene (ANOVA, $F_{1,90}$=154.88, P<0.0001) (Table 5.3). Surfaces treated with adjuvants had 100% adult emergence at each duration. For the methoprene-treated surfaces, there was a significant difference of duration on adult emergence (ANOVA, $F_{3,45}$=28.96, P<0.0001). However, there were no significant differences in adult emergence due to the presence of flour (ANOVA, $F_{1,45}$=1.40, P=0.2431) or cleaning frequency (ANOVA, $F_{2,45}$=2.45, P=0.0975). To investigate the main effect of cleaning protocol on adult emergence, two contrasts were constructed. The first showed that there was no significant difference in adult emergence between the surfaces which had flour and were not cleaned and those that had no flour (hence not cleaned) (ANOVA, $F_{1,45}$=3.06, P=0.0872). The second showed that adult emergence was significantly lower on surfaces with no flour compared to the average of all the other surfaces that had flour (some of which received cleaning) (ANOVA, $F_{1,45}$=5.95, P=0.0187). In this last comparison, the interaction of cleaning protocol x duration was not significant (ANOVA, $F_{9,45}$=0.68, P=0.7223).

Effect of high temperatures on residual efficacy

The concrete and varnished-wood surfaces treated with adjuvants had 100% adult emergence. The adult emergence on surfaces treated with adjuvants was significantly higher than on methoprene-treated surfaces (ANOVA, $F_{1,60}$=1557.41, P<0.0001). Adult emergence was significantly higher on concrete surfaces than on varnished-wood surfaces (ANOVA, $F_{1,60}$=44.04, P<0.0001). As there was no variation from zero in the adult emergence on varnished-wood surfaces treated with methoprene, only the data on concrete surfaces were used in the subsequent analysis. The adult emergence on concrete surfaces treated with methoprene did not differ with the duration at 65ºC (ANOVA, $F_{4,15}$=1.35, P=0.2694) (Table 5.4).

Effect of humidity on bioassay

The humidity level in the incubator that had adjuvants-treated surfaces (20.26±0.11%) was higher than that in the incubator which had methoprene-treated surfaces (16.29±0.02%) (ANOVA, $F_{1,4127}$=1349.2, P<0.0001). However these different humidity levels did not affect adult emergence (ANOVA $F_{1,19}$=0.42, P=0.5226) (Table 5.5). Furthermore, when the regular experiment and the new bioassay were compared, there were no significant differences in the adult emergence on concrete (ANOVA, $F_{1,6}$=1.0, P=0.3559) or varnished-wood surfaces treated with adjuvants. Similarly, there were no significant differences on concrete (ANOVA, $F_{1,4}$=0.19, P=0.6822) or varnished-wood surfaces treated with methoprene.

Residual efficacy of methoprene on wheat maintained at high temperature

Based on the contrast statements employed in analysis, there was no significant difference between water and adjuvants in terms of adult emergence (ANOVA, $F_{1,108}$=1.52, P=0.2207). However, there was a significant difference in adult emergence between the controls (water and adjuvants) and methoprene concentrations (ANOVA, $F_{1,108}$=66.27, P<0.0001). Therefore in the subsequent analysis, only the data with different methoprene concentrations were used to find the changes in the residual efficacy. The adult emergence significantly differed with the methoprene concentration (ANOVA, $F_{3,72}$=70.61, P<0.0001). However, there was no effect of duration at 46ºC (ANOVA, $F_{5,72}$=1.07, P=0.3818), or methoprene concentration x duration interaction (ANOVA, $F_{15,72}$=0.73, P=0.7496) on adult emergence, revealing that methoprene is stable for 48 h at 46ºC (Table 5.6, Figure 5.2).

Discussion

Efficacy of chemical control depends on a number of factors. The most important are dose of the insecticide (Arthur 1998b), its formulation (Arthur 1996), method of application (Arthur 1997a), surface material (Toews and Subramanyam 2003; Arthur 1997a), duration of exposure (Arthur 1998b), temperature (Hagstrum and Subramanyam 2006), cleanliness of the surface (Watters 1970) and insect species (Sparks et al. 1982, Arthur 1997a; Toews et al. 2003).

In these current experiments, residual efficacy of methoprene on concrete decreased with time, whereas on varnished wood it did not. There is little information on the degradation of methoprene on surfaces over time. Methoprene on cardboard paper

loses its efficacy by 23%, following storage at 30ºC for 50 days when bioassayed with *E. kuehniella* (Tan and Tan 1980). Degradation over time is also common in other grain insecticides (Snelson 1987). Many insecticides are less persistent on concrete (Williams et al. 1983; White and Leesch 1996; Hagstrum and Subramanyam 2006). There are several reasons for this. Concrete surfaces are porous (Arthur and Hoernemann 2004), and this enhances the penetration of sprayed insecticide into the surfaces leaving little insecticide on the surface to contact with insects. Accordingly, the efficacy of malathion on unpainted concrete surfaces is lower than painted surfaces (Burkholder and Dickie 1966). As a second factor, pH is high on concrete (approximately 10.5), and this increases hydrolysis of insecticides (White and Leesch 1996). Conversely, pH of wood is about 6.0, and consequently the duration of residual efficacy is longer than on concrete (White and Leesch 1996). When grain dust accumulates on concrete surfaces treated with malathion, the pH of the concrete is reduced and malathion remains longer on the surface extending its residual efficacy on *T. confusum* (Watters 1970). Methoprene is less effective at preventing the emergence of *P. interpunctella* when applied to concrete than when on wood or laminated paper (Jenson et al. 2009). The residual efficacy of pyrethroid insecticides against *T. castaneum* gradually declines over 33 weeks on plywood (Watters et al. 1983). Further research is needed to determine if the decline in the residual efficacy of methoprene on concrete surfaces over 24-week period can be attributed to its high porosity or high alkalinity. Alternatively, the varnished layer covering the wood surface may have acted as a physical barrier for the penetration of methoprene into the wood, and thus the presence of higher amounts of methoprene on its surface would have reduced the adult emergence.

Presence of flour decreased the residual efficacy on concrete surfaces, but not on varnished-wood surfaces. There are several possible explanations for this. It could be that the same amount of flour (7 g) put on concrete and varnished-wood surfaces removed equal amounts of methoprene, but the differential properties of surface type (such as less porosity on varnished wood) left more methoprene on the varnished-wood surface, compared to the concrete surface. The amount of methoprene left on varnished wood was enough to totally suppress the development of larvae, whereas the amount of methoprene left on concrete was not. Similar effects of flour on residual efficacy of insecticides applied on surfaces have been reported. On concrete sprayed with cyfluthrin and subsequently covered with flour, the survival of *T. castaneum* was increased with the increased amount of flour/area covered by flour (Arthur 2000). Also, the presence of flour on Petri dishes treated with pyrethrins or esfenvalerate increases survival of *T. castaneum* (Toews et al. 2010). In these cases, the possibilities for the reduced efficacy of insecticides by flour on surfaces are such that it provides a barrier for the contact of insects with insecticides, supplies nutrition to the insects, or removes insecticide on the insect body (Arthur 2000).

These experiments show that methoprene is stable from 20 to 35ºC over months, and at 65ºC for hours. This demonstrates that methoprene can be used under many different storage conditions, and storage practices such as heat treatments. This is an advantage, as generally insecticides have lower efficacy with increased temperature (Hagstrum and Subramanyam 2006). For example, the residual efficacy of chlorpyrifos methyl applied on wheat declines more when held at 35ºC than at 15ºC (Arthur et al. 1992). As shown in the current study, the residual efficacy of methoprene on wheat was

not reduced after 48 h at 46ºC. This agrees with earlier findings that methoprene is persistent. On peanuts and shelled corn, methoprene persists for a few months (Snelson 1987); on maize (Daglish et al. 1995) and sorghum (Daglish and Wallbank 2005) at 20-30ºC, methoprene remains effective for at least for 6 months following application. This indicates the feasibility of using methoprene in commodity heat treatments.

Excellent residual efficacy over six months, regardless of the presence of flour makes varnished wood a better surface for application of methoprene. However, the rapid decline in efficacy on unfinished concrete surfaces suggests that methoprene alone would not be a successful long-term control agent on concrete floors and walls. As reapplication of methoprene is expensive, alternatives are needed to increase residual efficacy on unfinished concrete. Mixing methoprene with some other insecticide that is effective on unfinished concrete may provide long-lasting control. Another option would be to seal the concrete with paint or varnish. This has proven effective for cyfluthrin (Arthur 1994a). Although residual efficacy on varnished wood was not reduced by flour, the presence of high amounts of flour would favor the population build up if larvae do not come into contact with the residues on the surface. In such conditions, cleaning of surfaces is required and future research is needed to determine if methoprene is removed by the combination of the presence of flour and cleaning on varnished wood.

This research provides information for the use of methoprene in warehouses, mills and processing plants. Methoprene is well-known as an IGR for decades, and it has been tested against many stored-product insect species. However, there has been little research to demonstrate how methoprene can be used under real storage conditions. Many of the experiments in this paper simulate potential practical applications of methoprene.

However, additional tests under real-world conditions are needed to verify the trends shown in these experiments.

Table 5.1. Percentage adult emergence (mean±SEM) from late-instar larvae of *Tribolium castaneum* held on methoprene-treated surfaces for 3 weeks at 30ºC (n=4, 20 larvae/replicate). Before introducing larvae, the surfaces were held at 20, 30 or 35ºC for 0-24 weeks.

Surface type	Temperature (ºC)	Percentage adult emergence (mean±SEM)					
		Duration (weeks)					
		0	8	12	16	20	24
Concrete	20	2.5±2.5	27.5±22.5	41.3±18.6	58.8±22.8	43.8±16.0	90.0±6.1
	30	2.5±2.5	16.3±16.3	37.5±18.0	42.5±8.8	17.5±17.5	48.8±26.8
	35	2.5±2.5	21.3±16.4	47.5±14.5	50.0±20.5	52.5±23.8	68.8±23.0
Varnished wood	20	0±0	0±0	0±0	0±0	0±0	0±0
	30	0±0	0±0	0±0	0±0	0±0	0±0
	35	0±0	0±0	0±0	0±0	0±0	0±0

Table 5.2. Percentage adult emergence (mean±SEM) from late-instar larvae of *Tribolium castaneum* held on methoprene-treated surfaces (with or without flour) for 3 weeks at 30ºC (n=4, 20 larvae/replicate). Before introducing larvae, the surfaces were held at 30ºC for 0-24 weeks.

Surface type	Presence of flour	Percentage adult emergence (mean±SEM)					
		Duration (weeks)					
		0	8	12	16	20	24
Concrete	Without	2.5±2.5	16.3±16.3	37.5±18.0	42.5±8.8	17.5±17.5	48.8±26.8
	With	5.0±5.0	96.3±3.8	100±0	100±0	92.5±7.5	100±0
Varnished wood	Without	0±0	0±0	0±0	0±0	0±0	0±0
	With	0±0	0±0	0±0	0±0	0±0	0±0

Table 5.3. Percentage adult emergence (mean±SEM) from late-instar larvae of *Tribolium castaneum* held on methoprene-treated concrete surfaces for 3 weeks at 30ºC (n=4, 20 larvae/replicate). Before introducing larvae, the surfaces were held at 30ºC for 0-16 weeks with different cleaning frequencies.

Presence of flour	Cleaning frequency per every four weeks	Percentage adult emergence (mean±SEM)			
		Duration (weeks)			
		0	4	8	16
Without	0	2.5±2.5	26.7±26.7[1]	71.7±25.9[1]	76.7±14.5[1]
With	0	5.0±5.0	58.8±22.9	76.3±12.8	87.5±7.5
With	1	5.0±5.0	61.3±24.2	82.5±12.7	96.3±2.4
With	4	5.0±5.0	88.8±8.3	100±0	100±0

1. Treatments had three replicates.

Table 5.4. Percentage adult emergence (mean±SEM) from late-instar larvae of *Tribolium castaneum* held on methoprene-treated surfaces for 3 weeks at 30ºC (n=4, 20 larvae/replicate). Before introducing larvae, the surfaces were held at 65ºC for 0-48 hours.

Surface type	Duration at 65ºC (h)	Percentage adult emergence (mean±SEM)
Concrete	0	23.8±8.3
	6	35.0±5.4
	12	22.5±12.7
	24	35.0±12.7
	48	10.0±10.0
Varnished wood	0	0±0
	6	0±0
	12	0±0
	24	0±0
	48	0±0

Table 5.5. Percentage adult emergence (mean±SEM) from late-instar larvae of *Tribolium castaneum* held on methoprene-treated surfaces for 3 weeks at 30ºC under different relative humidities (n=4, 20 larvae/replicate). Before introducing larvae, the surfaces were held at 35ºC for 24 weeks.

Surface type	Percentage adult emergence (mean±SEM)			
	Adjuvants		Methoprene	
	Relative humidity (%)		Relative humidity (%)	
	16.3	20.3	16.3	20.3
Concrete	100±0	98.8±1.2	68.8±22.9	85.0±0[2]
Varnished wood	100±0[1]	100±0	0±0	0±0[2]

1. Treatment had three replicates
2. Treatments had two replicates

Table 5.6. Percentage adult emergence (mean±SEM) from late-instar larvae of *Tribolium castaneum* held on methoprene-treated wheat for 3 weeks at 30ºC (n=4, 20 larvae/replicate). Before introducing larvae, wheat was held at 46ºC for 0-48 hours.

Concentration (ppm)	Percentage adult emergence (mean±SEM)					
	Duration (h)					
	0	3	6	12	24	48
0 (Water)	98.8±1.2	98.8±1.2	97.5±1.4	100±0	100±0	97.5±2.5
0 (Adjuvants)	98.8±1.2	100±0	98.8±1.2	97.5±1.4	100±0	98.8±1.2
0.003	100±0	98.8±1.2	100±0	100±0	100±0	100±0
0.00825	97.5±2.5	97.5±2.5	100±0	97.5±1.4	93.8±2.4	98.8±1.2
0.0165	95.0±3.5	96.3±2.4	96.3±3.8	88.8±4.3	97.5±1.4	96.3±1.3
0.033	77.5±5.2	76.3±4.3	76.3±3.1	72.5±5.2	76.3±3.8	82.5±5.9

Figure 5.1. Average adult emergence from *Tribolium castaneum* late-instar larvae introduced on concrete and varnished-wood surfaces treated with methoprene and held for different durations (n=12, 20 larvae/replicate). The data are pooled across three temperatures (20, 30 and 35ºC), as temperature had no effect.

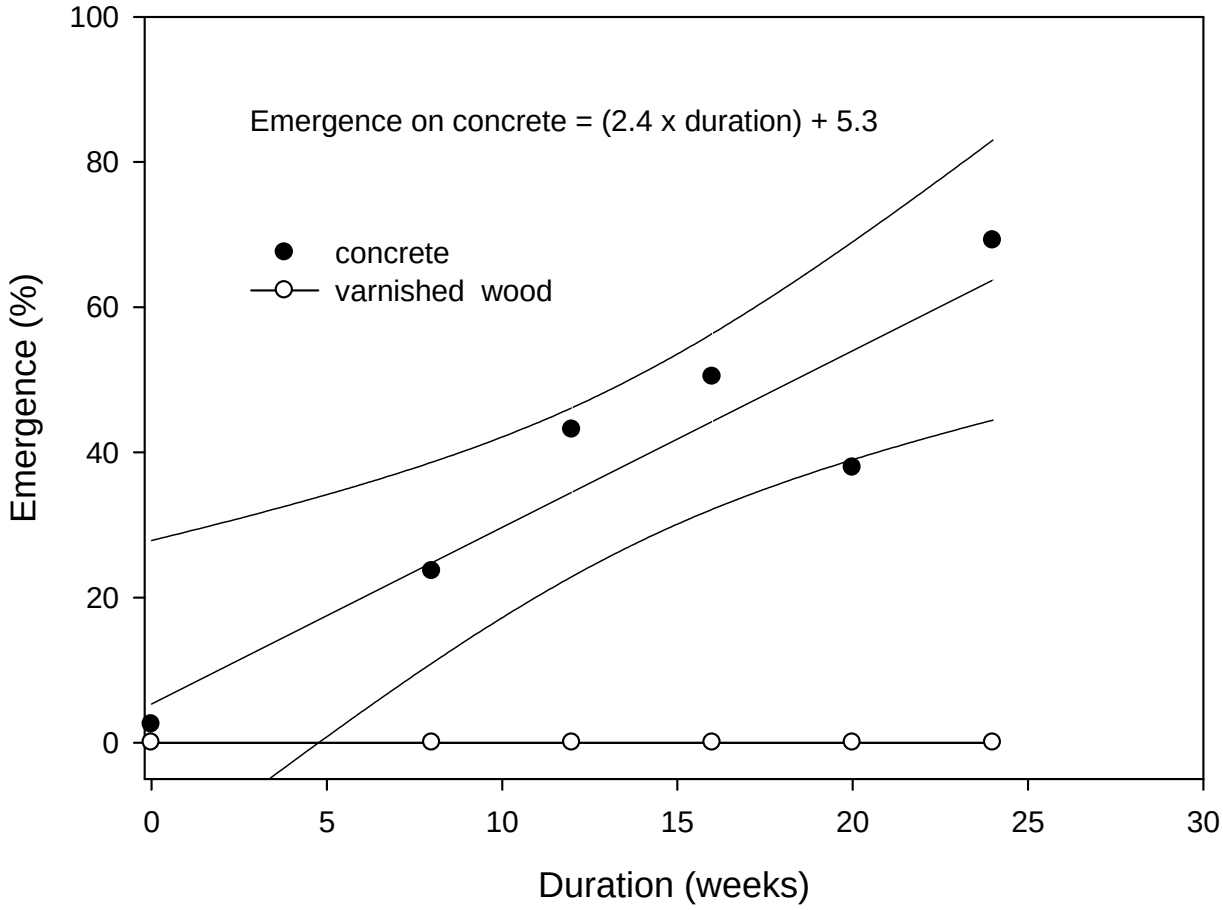
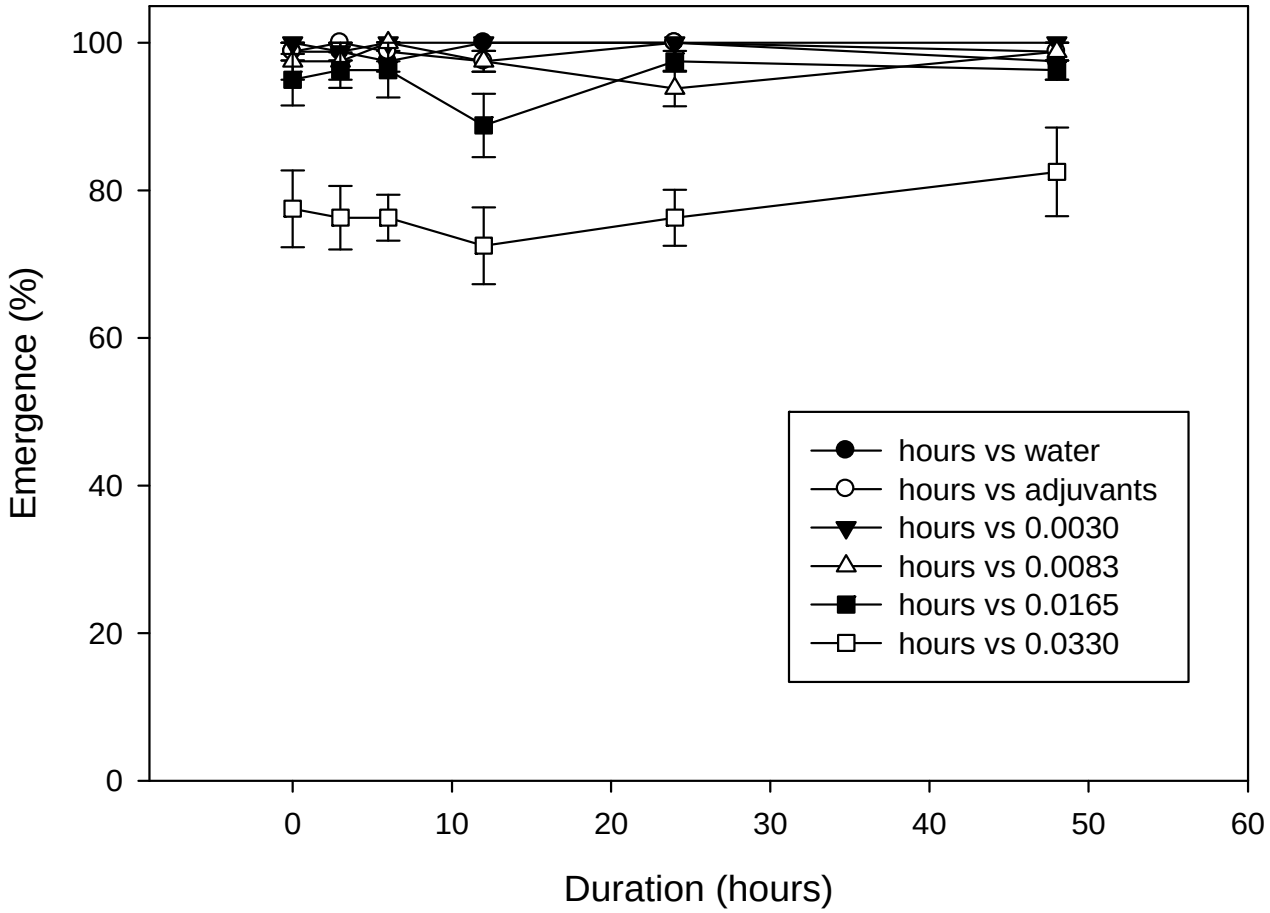


Figure 5.2. Percentage adult emergence (mean±SEM) from *Tribolium castaneum* late-instar larvae exposed to wheat treated with different concentrations of methoprene for 3 weeks. Before introducing larvae, wheat was held at 46ºC for 0-48 hours.



Chapter 6. General Discussion

This dissertation examines how the JHA methoprene interferes with heat tolerance, cold tolerance, progeny production of *T. castaneum*, and the use of methoprene as applied to surfaces. These experiments, in one way characterize the effects of methoprene on some selected physiological processes of *T. castaneum*. On the other hand, these findings can be used in the management practices for this species. Findings in the Chapter 3 reveal reduction in the heat tolerance of *T. castaneum* adults due to methoprene. Chapter 4 describes that *T. castaneum* larvae exposed to methoprene reduce their progeny production as adults. Chapter 5 outlines change in the toxicity of methoprene applied on surfaces to larvae, under different temperatures, surfaces or cleaning regimes. Therefore, it is important to understand how these findings can be linked to the practical situations in the stored-product environment.

The work outlined in this dissertation has implications for the two main uses of insecticides in stored products: controlling insects in commodities and controlling insects in structures (warehouses, empty bins and cereal processing facilities). Experiments on heat tolerance, cold tolerance and progeny production were conducted on a commodity (wheat). The residual efficacy of methoprene was assessed on concrete and varnished-wood surfaces; two types of surfaces commonly found the storage and processing facilities. The residual efficacy was also determined on wheat, one of the major commodities in the world.

Commodity treatments

There are a number of implications that can be derived from this study on using methoprene in commodity treatments. This study showed that methoprene applied on wheat reduces heat tolerance of *T. castaneum* adults (Chapter 3). Heat treatment of commodities has been developed as an alternative because of the concerns over neurotoxic insecticides in grain, the loss of fumigant methyl bromide and presence of phosphine-resistant populations (Kirkpatrick and Tilton 1972; Tilton et al. 1983; Beckett et al. 2007). Commodities are heated to 50 to 60ºC for minutes or seconds to control insects. Often the commodity must be precisely heated and rapidly cooled to avoid heat damage to the commodity. Reducing the heat tolerance of target insects with methoprene could make commodity-heat treatment more effective. Chapter 3 shows that exposure of *T. castaneum* adults to wheat treated with methoprene reduces their survival under heat treatment conditions; the effective concentration 3.33 ppm is within the current label rate (1-5 ppm) (Anonymous b 2011). Work with other commodities such as barley, maize, chickpeas, and other insect species is necessary to determine the effective concentration and efficacy on other species. Additional adjuvants, or synergists such as piperonyl butoxide (PBO) (Pospischil and Smith 1994) may allow the reduction of methoprene concentrations required to reduce heat tolerance. However, such an approach would require modification of the label, which is a long and costly process. Finally, testing at temperatures below 46ºC should also be done to determine if heat tolerance is reduced under such temperatures. Insects eventually die at temperatures as low as 40ºC (Fields 1992). The mechanisms of mortality at these low temperatures are due to desiccation rather than to the biochemical mechanisms described in the literature review (Chapter 2),

and this desiccation may change the sensitivity to methoprene when insects are under heat stress.

Methoprene on wheat at 27ºC is effective for 12 months against *R. dominica* and *S. oryzae* (Mian and Mulla 1982a, 1982b). The work in this thesis showed that methoprene is stable on wheat for 48 h at 46ºC (Chapter 5). This suggests that methoprene may still be effective after a commodity-heat treatment. Additional tests are required at the times and temperatures used with commodity-heat treatments to verify efficacy. Also, the measurement of methoprene residues, in addition to the insect bioassays, would give a more accurate picture of the loss of methoprene during commodity-heat treatment.

Methoprene did not reduce the cold tolerance of *T. castaneum,* nor did it block cold acclimation of *T. castaneum. Tribolium castaneum* does not have a diapausing stage. However, more than forty stored-product insect species (Dermestidae, Ptinidae, Bruchidae and Pyralidae) have a diapausing stage (Bell 1994). Some insect diapauses are regulated by JH (Yagi and Fukaya 1974; Chippendale 1977; Eizaguirre et al. 1998; Munyiri and Ishikawa 2004). Also, cold tolerance and diapause can be linked (Denlinger 1991). For example, diapausing larvae of *P. interpunctella* have higher cold tolerance than non-diapausing larvae (Naeemullah et al. 1999; Fields and Timlick 2010). Therefore, there is a possibility that methoprene may affect cold tolerance in some other stored-product species that have a diapausing stage.

Methoprene applied to larvae at 0.001 ppm caused approximately 37% mortality, and the surviving adults had much lower reproductive success (Chapter 4). This concentration (0.001 ppm) is 1600-5000 times less than the current label rate of

methoprene on wheat of 1-5 ppm (Anonymous b 2011), and suggests that the effectiveness of methoprene would be much longer than shown by Mian and Mulla (1982a, 1982b). Stored-product insects can remain undetected for months, but their high reproductive potential (Hagstrum and Subramanyam 2006) can cause them to rapidly increase under warm, humid conditions, causing extensive damage to the grain before grain managers realize that there is a problem. The current work shows that methoprene will continue to reduce insect populations, long after the methoprene residues fall below 1 ppm.

Methoprene on commodities can also be used with some other control method to have additive or synergistic effects. Examples of such combination of treatments are diatomaceous earth and silica areogel (Korunic and Fields 1998); the pyrethroid cyfluthrin and PBO (Arthur 1994b; Pospischil and Smith 1994); spinosad and chlorpyrifos-methyl (Daglish 2008). Diatomaceous earth absorbs lipids in insect cuticle, causing the insects to lose body water and die from desiccation (Ebeling 1971). Combination of methoprene and DE can be recommended for reducing progeny of *T. castaneum*. As described above, the residual efficacy of methoprene applied at the label rate decreases with time although it may still reduce the progeny production. In this case, diatomaceous earth can cause a lethal effect. Methoprene resistance has been detected in both stored-product insects (*L. serricorne*) (Benezet et al. 1993) and non stored-product insects (*O. nigromaculis*) (Cornel et al. 2002). The exact mechanism by which an insect becomes resistant to methoprene has not yet been determined (Palli 2009). However, resistance to methoprene in *Musca domestica* L. (Diptera: Muscidae) is believed to be caused by the increased oxidative metabolism (Hammock et al. 1977). Piperonyl butoxide

is an inhibitor of cytochrome P450 (Kennaugh et al. 1993), an enzyme used in detoxification metabolism (Darwish et al. 2010). The PBO synergizes pyrethroid (Pospischil and Smith 1994), therefore, it may be worthwhile to determine if PBO can synergize methoprene.

Structural treatments

Findings on the residual efficacy of methoprene applied on concrete and varnished-wood surfaces reveal a number of possible practical applications; some of them can be directly used, whereas others would need modifications. Methoprene applied at the label rate on varnished wood suppressed the development of *T. castaneum* larvae for at least 24 weeks. In contrast, methoprene applied to unfinished concrete had 20% of larvae emerged as adults after 8 weeks. Temperatures between 20 and 35ºC did not affect rate of degradation with time over the 24 weeks. Further research on the factors that affect the breakdown of efficacy on concrete such as porosity, pH and type of concrete may help to find adjuvants that could prevent this degradation.

This study shows that varnished wood is an excellent surface in storage structures for methoprene application. However, this work on surface application should be expanded to include a wider range of surfaces typically found in warehouses, processing facilities and also various packing material. Some of these include finished concrete, cinder blocks, metal, paper, jute, polythene and wood. This would enable a better prediction of the necessary reapplication times for methoprene. Also, tests with longer durations are required to determine when reapplication is required for varnished wood, which completely suppressed the development of *T. castaneum* larvae even after 24

weeks. Further testing with different temperatures is probably not needed as this was found not be a factor in the degradation as shown with both unfinished concrete (degradation occurred) and varnished wood (no degradation occurred).

Another possible use of methoprene as a surface application is its use in structures where heat is used to control insects within the buildings and equipments. In structural heat treatments, a temperature of 50-60ºC is maintained for 24-36 h (Dosland et al. 2006; Beckett et al. 2007). My work showed that methoprene did not lose efficacy on concrete and varnished wood even after 48 h at 65ºC (Chapter 5). Therefore, reapplication of methoprene right after a heat treatment is not needed. Further research needs to verify that these trends are seen with other surfaces. Adults of *T. castaneum* exposed to methoprene-treated wheat had lower heat tolerance (Chapter 3). This work should be repeated with different types of surfaces to determine if methoprene applied on surfaces reduces heat tolerance of *T. castaneum* and other species, so that this technology could be used in structural heat treatments.

Finally, similar to that proposed with commodity applications, methoprene on surfaces also need to be tested with other insecticides for possible additivity or synergy. Diatomaceous earth and other insecticides are possible agents on surfaces, as on commodities. Surface application of methoprene on varnished wood will control larvae. However, *T. castaneum* larvae are more heat tolerant than adults (Mahroof et al. 2003) and they may survive following heat treatments. These surviving larvae from a heat treatment may seek food sources, continue feeding, and perhaps emerge as adults. However once the larvae are exposed to sub-lethal doses of methoprene, the emerged adults may have impaired reproduction. Similar to the suggestion with commodity

treatments, DE may kill these immatures, which may survive heat treatments, as well as adults.

Additional research

Based on the findings of the experiments in this dissertation, additional research is recommended in several areas. The current work only looked at the effects of methoprene on *T. castaneum*. There are several hundreds of insect species that infest stored products (Hagstrum and Subramanyam 2006). The effects of insecticides on stored-product insects are quite variable. For example, DE is more toxic to *C. ferrugineus* than to *T. castaneum* (Fields and Korunic 2000). The residual efficacy of deltamethrin dust on wood, concrete and tile surfaces is more toxic for *T. castaneum* than *T. confusum* (Arthur 1997a). Grain and warehouse managers need to control all the stored-product species present. The most promising work would be to verify if methoprene also reduces the heat tolerance of other insects. The effect of methoprene on progeny production was very pronounced in my research. Insects with different reproductive strategies, such as the short-lived pyralidae moths and the bruchids may have different development of their reproductive systems, and hence the effect of methoprene could be different from that on *T. castaneum*. In addition to the pest insects, this work could be expanded to examine the effects of methoprene on parasitoids of stored-product insects (McNeil 1975).

This work only examined the effects of methoprene on *T. castaneum*, and that the effects of methoprene on insects are more than just the prevention of larval development to adults. However there are several JHAs and IGRs that are used commercially to control insects. Pointsource™ (Arthur 2003), Gentrol® (Arthur 2001) and Protrol® (Bell

and Edwards 1999) are commercial products of hydroprene and NyGuard® is a pyriproxyfen (Arthur and Phillips 2009). Tebufenozide (Mimic) is an ecdysteroid analogue (Oberlander et al. 1995). The commercially available chitin synthesis inhibitory products are Novaluron (Kostyukovsky and Trostanetsky 2006), Dimilin (diflubenzuron) (Oberlander and Silhacek 2000), Atabron (chlorfluazuron) and Nomolt (teflubenzuron) (Ishaaya et al. 2003). Within the JHAs, the effects may sometimes differ between compounds (Lim and Lee 2005; White et al. 1987; Edwards and Short 1984). The effect of reduced heat tolerance may also be seen with other JHAs.

Suppression of heat shock proteins in *T. castaneum* adults was proposed as a possible mechanism for the reduced heat tolerance observed in adults (Chapter 3). Both larvae and adults produce heat shock proteins in response to exposure at high temperature (Mahroof et al. 2005b), however only the adults had reduced heat tolerance after exposure to methoprene. Larvae are very sensitive to methoprene, so any effect of reduced heat tolerance by methoprene may be undetectable compared to its large effects against development. Testing the effect of methoprene on the production of heat shock proteins in both larvae and adults may help to understand the mechanism responsible for the reduced heat tolerance in adults. For this experiment, spraying wheat with methoprene or adjuvants, exposure of *T. castaneum* adults or larvae on treated wheat and subsequent exposure to heat (46ºC) for different durations can be conducted as in the previous experiment (Chapter 3). Three genes that encode HSP 70 in *T. castaneum* have been identified (*tchsp 70 I*, *tchsp 70 1I* and *tchsp 70 11I*) (Mahroof et al. 2005b). Therefore, the adults or larvae that survive heat exposure can be used to detect if there is a difference in the up regulation of those genes between methoprene-treated and control

(treated with adjuvants) insects exposed to 46ºC. This study would also enable determination of whether there is a difference in the expression of those three genes in the presence of methoprene and if so, which genes are more responsible to reduce heat tolerance. The study to find out mechanism of reduced heat tolerance could be expanded to include other compounds, such as trehalose (Singer and Lindquist 1998), glycerol or sorbitol (Denlinger and Yokum 1998; Wolfe et al. 1998), amino acids (Malmendal et al. 2006) or dopamine (Rauschenbach et al. 1993) that are associated with heat tolerance in insects or other organisms.

The mechanism by which methoprene reduced progeny production (Chapter 5) still needs to be determined. Possible mechanisms are disruption of spermatogenesis (Dumser and Davey 1974); disruption of proper functioning of accessory glands (Bodenstein and Sprague 1959; Koeppe et al. 1985); effect on the development of oviducts, follicles or oocytes (Koeppe et al. 1980; Koeppe et al. 1985); and/or effects on pheromone production (Kim et al. 2005) or mating (Segura et al. 2009).

Summary

This dissertation offers valuable contributions for stored-product insect pest management programs. Reduction of heat tolerance in *T. castaneum* adults by methoprene is an innovative finding, as there are no previous studies that documented this effect using JH or a JHA for any other insect species. The study on the effect of methoprene on progeny production will help to understand that methoprene reduces *T. castaneum* populations even at very low concentrations that materialize during degradation. Residual efficacy experiments offer several recommendations for how to use

methoprene under various storage and processing conditions. The findings in this dissertation should enable better use of methoprene to control *T. castaneum* both in commodities and in structures. It has also provided some insights into the possible roles of JH in the regulation of heat tolerance in adults and the development of reproductive systems of male and female *T. castaneum*. These would also serve as areas for further research to augment the knowledge on insect physiology.

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Appendix

In the initial stages of my Ph.D. dissertation, I intended to examine the effects of methoprene on both *T. castaneum* and *P. interpunctella*. The rationale for selecting these two species was that they represent two widely different taxonomic groups of stored-product insects, and they have very different physiology. As *P. interpunctella* has a larval diapause, one of the early objectives was to determine if methoprene affects its diapause induction. As the first step of this study, experiments were launched to obtain a population that is capable of diapausing, by using four *P. interpunctella* populations from different geographical regions. As the frequency of diapause induction was not high enough for testing with methoprene, the experiments were setup to select for a higher frequency of diapause in one population. Although there were promising results with diapause selection, this research had already taken two and a half years of the study program. In consultation with my Ph.D. advisory committee, I decided to stop further experiments on *P. interpunctella*, and only study the effects of methoprene on *T. castaneum*. This appendix reports the work completed on diapause induction in *P. interpunctella*.

Effects of rearing conditions, geographical origin and selection on larval diapause in *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae)

Abstract

Plodia interpunctella, the Indianmeal moth, is a serious insect pest of stored products and its late-instar larva diapauses as a pre-pupa. Diapause induction in *P. interpunctella* was investigated for four populations obtained from Modesto, California, U.S.A.; Vancouver, British Columbia, Canada; and two locations from Winnipeg, Manitoba, Canada. Insects were reared at 25ºC and 16 h light: 8 h dark for 9 days. They were then either continuously maintained under those conditions or transferred to 25ºC, 8L: 16D; 20ºC, 16L: 8D; or 20ºC, 8L: 16D, and the percent diapause recorded. In a second experiment, the Vancouver population was selected for diapause. Larvae were reared at 25°C, 16L: 8D for 9 days, then placed at 20°C, 8L: 16D for the rest of their development, and percent diapause determined. Eggs laid by moths that completed diapause in this first (parental) generation were used to obtain a second generation (F_1) and the experiment was repeated as in the first generation.

In the experiment with four populations, the highest diapause frequency was observed at 20ºC, 8L: 16D. The two Winnipeg populations had significantly higher frequency of diapause than California population, indicating the increased frequency of diapause in populations from higher latitudes. In the selection experiment on the Vancouver population, selection increased the frequency of diapause to 91% compared to 26% in the unselected population.

Introduction

Indianmeal moth, *P. interpunctella* is an insect pest species of stored products that is found throughout the world (Rees 2004). It causes infestations in a wide array of commodities (Johnson et al. 1992; Johnson et al. 1995; Sedlacek et al. 1996; Nansen and Phillips 2003; Nansen and Phillips 2004; Nansen et al. 2004). This species undergoes larval diapause (Bell and Walker 1973) as a pre-pupa (Prevett 1971). Diapause induction in *P. interpunctella* depends on a number of factors; photoperiod (Bell and Walker 1973; Kikukawa et al. 2005), temperature (Tsuji 1958; Tzanakakis 1959; Prevett 1971; Bell 1976a; Mbata 1987), age of larvae exposed to diapause-inducing conditions (Bell 1976a), origin of population (Bell 1976a), larval density (Tsuji 1959a; Tsuji 1960) and type of food (Williams 1964).

There have been previous studies that explored the effects of ecological factors on diapause induction in *P. interpunctella* (Tsuji 1958; Tsuji 1959a; Tsuji 1959b; Bell 1976a; Mbata 1987). Some of these studies examined the effect of temperature and light on the diapause induction in this species (Bell et al. 1979; Mbata 1987). Information on the response of diapause frequency to selection is scarce for *P. interpunctella*. Hence, the objectives of this experiment were to study the effect of temperature and photoperiod on diapause induction in four populations of *P. interpunctella* from geographically different regions and to examine the effect of selection on frequency of diapause using one of the populations.

Methods

Diapause induction

Larvae of *P. interpunctella* populations from Modesto, California, U.S.A. (37° 38' 21" N / 120° 59' 44" W); Vancouver, British Columbia, Canada (49° 15' 0" N / 123° 8' 0" W); and two locations from Winnipeg, Manitoba, Canada (49° 53' 0" N / 97° 10' 0" W) were used in this experiment. Insects were reared at 25ºC, 16 h light, 8 h darkness (16L: 8D) on a rearing medium prepared by mixing 1000 g cracked wheat, 40 g brewer's yeast (ICN Biomedicals, Inc., Aurora, Ohio, U.S.A.), 50 g wheat germ, 2 g sorbic acid (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.), 2 g methyl-p-hydroxybenzoate (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.), 120 mL honey, 120 mL of glycerol and 60 mL of water. To collect eggs, fifty unsexed adults were placed in an empty 900 mL glass bottle, covered with a nylon mesh and placed upside down on a plastic Petri dish (Bell 1976a). Eggs were inspected under a microscope, and only plump eggs were used. Eggs collected within 24 h period were placed in batches of 50 eggs in glass bottles of the same dimensions with 200 g of rearing medium.

The developing insects were maintained at 25ºC and 16L: 8D for 14 days, before the bottles were placed in one of 4 conditions: 25ºC with 16L: 8D, 25ºC with 8L: 16D, 20ºC with 16L: 8D or 20ºC with 8L: 16D. Eggs took 4 to 6 days to hatch at 25ºC, 16L: 8D; therefore, larvae were approximately 9 days old at the time of transfer. Larvae developed under the above conditions and adults emerged. Seven days following the termination of this adult emergence under each condition, the rearing media were dissected and the presence of pre-pupae, dead larvae, dead pupae or dead adults was recorded. Live pre-pupae were considered to be in diapause.

Diapause selection

Only the Vancouver population was used in this experiment. Bottles with 50 eggs in 200 g of the medium were held at 25ºC, 16L: 8D for 14 days before they were transferred to 20ºC, 8L: 16D and held until emergence of non-diapaused adults was assumed to be completed. The adults emerged were counted and removed every 2-3 days. Ten days after the last adult emerged, the bottles were placed at 2.5ºC in total darkness and held for 6 weeks to induce termination of diapause of pre-pupae in the rearing medium (Bell 1976b). Following this 6-week exposure, bottles were returned to 25ºC, 16L: 8D and adults that subsequently emerged were counted and considered to have undergone diapause. These adults were held in empty glass jars, and their eggs (F_1) were collected and tested for diapause induction as described for the parental generation. Eggs from the adults that completed diapause in the F_1 (second) generation were used to start an F_2 (third generation). Frequency of diapause in this F_2 (third) generation was compared with that in unselected progeny derived from the original (Vancouver) population that was maintained continuously at 25ºC, 16L: 8D. Frequency of diapause in selected and unselected groups was investigated using the methods previously described involving exposure to four sets of conditions: 25ºC, 16L: 8D; 25ºC, 8L: 16D; 20ºC, 16L: 8D or 20ºC, 8L: 16D. As before, seven days after adult emergence ceased in each rearing jar, the rearing media were dissected.

Data analysis

The proportion of insects in diapause in each replicate was transformed using the square root of arcsine transformation. These transformed data were used in ANOVA (SAS Institute 2002-2008). In the diapause induction experiment, the effects of treatment (combination of temperature and photoperiod) and population (Vancouver, California, Winnipeg 1 and Winnipeg 2) on diapause induction were analyzed. For a given population, the means of the proportion of diapausing insects under different treatments were compared using Tukey's multiple range test or Student's t-test (P=0.05). In the selection experiment, the effect of selection on diapause frequency in the F_1 and F_2 generations were analyzed using ANOVA. An ANOVA was also performed to compare final diapause frequency in selected and unselected groups and this was followed by Tukey's multiple range tests (P=0.05).

Results

Diapause induction

The proportion in diapause significantly differed among treatments (ANOVA, $F_{3,28}$=88.3, P<0.0001) and among populations (ANOVA, $F_{3,28}$=6.54, P=0.0017). The interaction of treatment x population was also significant (ANOVA, $F_{9,28}$=5.18, P=0.0004). In all the populations, maximum diapause frequency was observed at 20ºC, 8L: 16D (Table 1). In the Vancouver and Winnipeg 1 populations, diapause frequency at 20ºC, 8L: 16D differed significantly from that in all other conditions. In the Winnipeg 2 and California populations, frequency of diapause was highest at 20ºC, 8L: 16D, but was not significantly different from all other treatments.

There were no significant differences in diapause frequency by decreasing only the photoperiod from 16 to 8 h at 25ºC (Tukey's test, Table 1). Similarly, decreasing only the temperature from 25 to 20ºC without decreasing the photoperiod from 16 h did not significantly change the diapause frequency. However, when both the temperature and photoperiod were reduced (from 25ºC, 16L: 8D to 20ºC, 8L: 16D), there was significant increase in diapause frequency in all four populations. Thus, lowering only the temperature or photoperiod did not significantly influence diapause frequency, but the operation of both factors concurrently did.

When only data from 20ºC, 8L: 16D were analyzed, significant differences among populations originated in different geographical locations were evident (ANOVA, $F_{3,7}$=6.20, P=0.0220). According to Tukey's mean separation, diapause frequency in Winnipeg 1 and Winnipeg 2 was significantly different from California population (P<0.05). However, diapause frequency in Vancouver population was not significantly different from any of the other three populations (P>0.05).

There was 11 to 68% of the insects that were unaccounted, or in other words that did not mature to late-instar larvae. This could be due to infertile eggs or mortality in the early instars that would not be detected during dissections.

Diapause selection

There was a significant change in percentage of adults emerging after cold treatment from the F_1 to the F_2 generation (ANOVA, $F_{1,33}$=58.82, P<0.0001) (Table 2). When this selected population was tested with the unselected original population, only at 20ºC, 8L: 16D there was a significant diapause induction. Under these conditions, the

selected population gave the highest percent diapause to date with 91.2%, which was higher than the unselected population (25.9%) (ANOVA, $F_{1,6}$=44.78, P=0.0005) (Table 3). There were no differences at 20ºC, 16L: 8D (ANOVA, $F_{1,6}$=2.98, P=0.1350); 25ºC, 16L: 8D; or 25ºC, 8L: 16D. ANOVA could not be conducted in the last two treatment conditions as the diapause did not change from zero. This shows that selection increased diapause in *P. interpunctella*.

Discussion

In the experiment with four populations, diapause induction mostly took place at 20ºC, 8L: 16D. This was also seen in the experiment in which the selected and unselected Vancouver populations were compared. Bell et al. (1979) showed that in 6 out of 23 populations have at least 20% diapause, when 2-day old larvae are transferred from 25ºC, 15L: 9D to 20ºC with 15L: 9D. The diapause frequency increases to 78% when the photoperiod is reduced to 11L: 13D. This is similar to my finding that the maximum diapause induction took place when late-instar larvae were transferred from 25ºC with 16L: 8D to 20ºC with 8L: 16D, although the diapause frequency varied from 7 to 34% among four populations. Bell et al. (1979) found that there is no diapause induction at 25ºC, 15L: 9D, and some diapause in a few populations at 25ºC, 9L: 15D. Similarly in the four populations I tested, diapause frequency was negligible both at 25ºC, 16L: 8D (0-1.2%) and 25ºC, 8L: 16D (0-0.6%). Mbata (1987) showed that the transfer of *P. interpunctella* fourth instar larvae from 30 to 20ºC give different diapause frequencies based on the photoperiod they are exposed; no diapause under continuous light and 37% under complete darkness.

In this study, in a given population, reducing only the photoperiod from 16 to 8 h at 25ºC did not change the diapause status. Also, in a given population, the diapause frequency was not changed by changing only the temperature (transferring larvae from 25ºC, 16L: 8D to 20ºC, 16L: 8D). In contrast, three out of four populations (Vancouver, Winnipeg 1 and Winnipeg 2) increased diapause frequency when both the temperature and photoperiod were decreased. Although not significantly different, the fourth population (California population) also had a trend to increased diapause frequency due to a similar change in temperature and photoperiod. This shows that the decrease in either photoperiod or temperature alone does not trigger diapause frequency in *P. interpunctella*, but requires the concurrent change in both factors. There have been some studies that used similar conditions to induce diapause in *P. interpunctella*, as tested in the current study. *Plodia interpunctella* larvae reared at 25ºC, 16L: 8D are induced to diapause when transferred to 20ºC, 8L: 16D; the percentage enters diapause vary with the age at which the larvae are transferred (Bell 1976a). In a later study, Bell et al. (1979) tested the effect of change in both the temperature and photoperiod independently and in combination. However in that study, the larvae were transferred to the new ecological conditions within relatively a short time following hatching (approximately in two days). In my study the larvae were transferred approximately 9 days after hatching, where they experience an ecological difference during the course of their life.

The populations from a higher latitude, Winnipeg, had greater diapause frequency than the population from a lower latitude, California. The population from Vancouver was not significantly different than California but showed similar diapause frequency as Winnipeg. This is in agreement with Bell et al. (1979), which showed that populations

from higher latitudes have higher frequencies of diapause than populations from lower latitudes.

Different criteria have been used to determine the diapause status in *P. interpunctella*. Mbata (1987) used the time taken from egg hatch to adult emergence as a criterion for diapause (23-34 d at 30ºC and 60-72 d at 20ºC as the non-diapausing condition). Tsuji (1958) recorded fully-grown arrested larvae as the indicator of diapause, compared to the non-diapausing larvae which pupated quickly. Bell (1976a) considered the larvae that had fully grown but did not pupate within two weeks to be in diapause. These differences in the criteria for determining the diapausing status may have contributed to the variation in interpretation of results to some extent.

Tsuji (1960) studied the effect of selection for diapause in *P. interpunctella* larvae. The study reports that *Plodia interpunctella* reared at 30ºC has 24% diapause when first collected from the field. This is declined to 1% after 3 years of continuous rearing at 30ºC. When larvae are reared at 20ºC with 11L: 13D, most enter diapause. By selecting the few individuals that do not enter diapause over several generations, the diapause frequency drops to 0.4% when reared at 20ºC with 11L: 13D. Selecting for diapausing individuals, maintains a population with almost all individuals entering diapause at 20ºC with 11L: 13D. In the current study on diapause selection, *P. interpunctella* larvae reared at 25ºC with 16L: 8D, induced to diapause at 20ºC with 8L: 16D, chilled at 2.5ºC, and terminated diapause at 25ºC with 16L: 8D increased diapause frequency from 18.8 to 50.2% in the two generations followed by an increase from 50.2 to 91.2% in a third generation. This suggests that in *P. interpunctella*, the frequency of diapause can be increased through selection.

Selection of a diapausing population does not always increase the frequency of diapause. At 20ºC, selection for diapause of an *E. cautella* diapausing population for three generations gives more or less constant diapause frequency between 26 and 29% (Cox et al. 1981). *Diatraea grandiosella* has a pupal diapause. Selection of that species at 30ºC increases the percentage diapause within three generations in the diapausing population from 56 to 100%. In non-diapausing population, the percentage diapaused decreases within four generations from 14 to 7% (Takeda and Chippendale 1982). Examples from these two pyralid relatives of *P. interpunctella* reveal the variety of response patterns in the diapause selection in different species. This nature of variation in response to diapause selection may be due to the presence of different genetic systems that regulate diapause in insects and only some respond quickly to selection (Danks 1987).

This study has shown that both low temperature and short photoperiod are required to induce diapause in North American populations of *P. interpunctella*. Practically this combination of conditions simulates the decreasing photoperiod in the fall season in temperate regions, during which *P. interpunctella* larvae enter diapause anticipating the forthcoming winter. Knowledge on differences in diapause induction under different ecological conditions will be important for comprehending the risk of the establishment of tropical *P. interpunctella* populations in temperate regions, through commerce. The diapause selection experiment reveals that diapause frequency can be increased within a few generations, at low temperatures and short photoperiod, indicating that the temperate countries need to adopt precautions to protect their stored products from this species. Therefore, renewed attention needs to be paid to diapause phenomenon

in *P. interpunctella* and research should be addressed on the management practices for this species.

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Table 1. Percent diapausing and non-diapausing *Plodia interpunctella* from different geographical populations.

Population	Temperature (ºC)	Day length (h)	Diapausing[4,6] (%) (mean±SEM)	Non-diapausing[4]			Unaccounted[5] (%) (mean±SEM)
				Adult emergence (%) (mean±SEM)	Live pupa (% (mean±SEM)	Total dead (%) (mean±SEM)	
California[1]	25	16	0.9±0.9ab	68.6±4.6	0±0	30.5±5.1	30.7±3.7
	25	8	0±0b	74.6±9.0	0±0	25.4±9.0	37.3±16.0
	20	16	1.7±1.7ab	62.8±8.8	2.4±2.4	33.1±6.9	25.3±10.0
	20	8	6.8±2.1a	76.2±3.7	0±0	17.0±4.5	30.7±10.9
Vancouver[2]	25	16	0±0b	90.4±2.4	1.2±1.2	8.4±2.7	17.5±3.6
	25	8	0.6±0.6b	87.8±2.7	3.9±2.0	7.8±1.6	24.5±4.9
	20	16	0±0b	94.0±3.9	0±0	6.0±4.0	57.0±6.8
	20	8	26.0±5.2a	71.5±4.8	2.5±2.5	0±0	68.0±5.9
Winnipeg 1[3]	25	16	0±0b	93.7±1.8	6.3±1.8	0±0	19.0±7.0
	25	8	0±0b	90.4±9.6	0±0	9.6±9.6	38.0±10.0
	20	16	0±0b	97.1±2.9	2.9±2.9	0±0	22.0±8.0
	20	8	34.6±10.8a	63.0±8.4	2.4±2.4	0±0	46.0±12.0
Winnipeg 2[3]	25	16	1.2±1.2bc	86.5±0.8	2.1±2.1	10.2±1.7	11.0±5.0
	25	8	0±0c	90.5±0.5	4.7±1.4	4.8±1.8	37.0±3.0
	20	16	10.1±1.0ab	88.5±2.4	0±0	1.4±1.4	31.0±3.0
	20	8	33.6±6.4a	60.4±3.2	3.0±3.0	3.0±0.1	32.0±2.0

1. Number of replicate jars=3, 50 eggs per replicate
2. Number of replicate jars=4, 50 eggs per replicate
3. Number of replicate jars=2, 50 eggs per replicate
4. Calculated as percentages of total number of individuals that could be accounted for in sample.
5. Calculated as;

$$\frac{\text{(total number of eggs introduced (50)} - \text{total number of individuals emerged)}}{\text{total number of eggs introduced (50)}} \times 100$$

6. For a given population, means followed by the same letter are not significantly different in diapause induction (Tukey's test, P=0.05).

Table 2. Percent diapausing and non-diapausing (mean±SEM) *Plodia interpunctella* (Vancouver population) reared at 25ºC with 16L: 8D for 14 days, transferred to 20ºC, 8L: 16D, terminated diapause by exposure at 2.5ºC for 6 weeks and selected over generations.

Generation	Diapausing[1,3]	Non-diapausing[1]		Unaccounted[2] (%) (mean±SEM)
	Adults emerged (after cold) (%) (mean±SEM)	Adults emerged (before cold) (%) (mean±SEM)	Dead (all life stages) (%) (mean±SEM)	
First[4]	18.8±3.0b	61.4±4.9	19.8±2.3	25.3±1.9
Second[5]	50.2±2.0a	8.5±1.3	41.3±2.3	35.9±3.8

1. Calculated as percentages of total number of individuals that could be accounted for in sample.
2. Calculated as;
   $$\frac{\text{(total number of eggs introduced} - \text{total number of individuals emerged)}}{\text{total number of eggs introduced}} \times 100$$
3. Means followed by the same letter are not significantly different (Tukey's test, P=0.05).
4. Number of replicate jars =16, 50 eggs per replicate
5. Number of replicate jars =19, 50 eggs per replicate

Table 3. Final comparison for diapause induction between selected progeny of second generation (Table 2) of Vancouver population and unselected original population (n=4).

Population	Temperature (ºC)	Day length (h)	Diapausing[1,3] (%) (mean±SEM)	Non-diapausing[1]			Unaccounted[2] (%) (mean±SEM)
				Adult emergence (%) (mean±SEM)	Live pupa (%) (mean±SEM)	Total dead (%) (mean±SEM)	
Unselected	25	16	0±0	91.7±1.7	0±0	8.3±3.2	14.0±2.6
Selected	25	16	0±0	91.3±3.2	0±0	8.7±3.2	31.4±6.4
Unselected	25	8	0±0	88.0±1.4	0±0	12.0±1.4	21.0±3.7
Selected	25	8	0±0	95.9±1.6	0±0	4.1±1.6	32.0±4.2
Unselected	20	16	0±0a	89.3±2.8	1.9±1.3	8.74±3.2	15.5±3.5
Selected	20	16	1.5±0.8a	87.6±3.0	0±0	10.9±2.8	22.5±6.1
Unselected	20	8	25.9±5.7b	69.1±6.4	0.8±0.4	4.2±2.3	24.5±5.9
Selected[4]	20	8	91.2±4.0a	2.3±2.3	6.5±2.7	0±0	27.0±6.2

1. Calculated as percentages of total number of individuals that could be accounted for in sample.
2. Calculated as;
   $$\frac{\text{(total number of eggs introduced} - \text{total number of individuals emerged)}}{\text{total number of eggs introduced}} \times 100$$
3. For a given temperature and light duration, means followed by the same letter (for unselected and selected populations) are not significantly different (Tukey's test, P=0.05).
4. This is the third generation of selected population.