HORIZONTAL TRANSFER OF METHOPRENE AND ITS EFFECT ON *TRIBOLIUM CASTANEUM* (HERBST) INDIVIDUALS AND POPULATIONS

by

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B.S., Kansas State University, 2003

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Aerosol applications of reduced risk insecticides such as synergized pyrethrin and insect growth regulators (IGR) are part of food industry integrated pest management programs. Since aerosols cannot penetrate into hidden areas exploited by pests such as the red flour beetle, *Tribolium castaneum*, the potential for these insecticides to effect beetle populations was evaluated. Because IGRs do not cause immediate mortality, the potential of horizontal transfer for an IGR from treated to untreated individuals was also examined. Results showed that when untreated *T. castaneum,* larvae or pupae, were added to flour containing methoprene, IGR, treated larvae, pupae or adults, the untreated individuals exhibited evidence of methoprene exposure (external deformities and reduced survival). Evaluation of the different mechanisms of transfer indicated that contact with methoprene treated individuals or flour that had been in contact with treated individuals may be the primary method of methoprene transfer. Since aerosols are often applied as a combination of IGR and pyrethrin with a carrier, the effect of these components was evaluated. Applications of synergized pyrethrin caused knockdown of adults but affected adults recovered and progeny production was not effected. Exposure of eggs to these insecticides reduced egg hatch. Food material accumulations inside food facilities can potentially increase or reduce insecticide efficacy. Evaluation of different flour residue levels, representing different sanitation levels, revealed that sanitation alone reduced immature development. As flour residue depths increased more individuals developed into adults but very few developed in the insecticide treatments. Food facilities that use aerosol insecticides apply them at regular intervals, so the cumulative effects of these treatments were considered. Experiments evaluating repeated insecticide exposures indicated that the direct morality from synergized pyrethrin not the horizontal transfer of methoprene was the primary factor in population reduction. Overall findings suggested that methoprene is highly mobile between different surfaces. Exposure of untreated individual beetle larvae to treated larvae or pupae or to flour that has been in contact with exposed beetles can have detrimental effects on development or survival, but these effects may be highly variable and even in cumulative exposures the overall level of population suppression is limited.

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Dedication

This body of work is dedicated to a little girl who had a dream and her father for encouraging her to dream “big.”

To my sisters; Vanessa Leach, Anne Lawrence, Nikki Malone DVM, and Bonnie Gilligan; your love, support, willingness to accept me as I am, and welcoming me into your families means more to me than a simple thank-you can convey.

To Jeff, in your short life you touched the lives of all who knew you but you also “lived” in every moment. Now that I have accomplished the “dream” I hope that I can get on with “living,” enjoying life, and fulfilling the next “big” dream!

Lastly, Judy, my experiences with you made me realize that family is more important than earning money or getting an education; your example has encouraged me to fight for a better life and to strive to be better than I was yesterday.

Chapter 1 - Introduction

Presence of stored product pests in post-harvest facilities can cause economic losses. The economic loses result from costs to manage insect pests and/or the presence of whole insects or insect fragments (Baur, 1984; Campbell and Arbogast, 2004; Sutton et al., 2011). Methyl bromide has historically been used to fumigate facilities to control stored product pests. However, in 1992 the Montreal Protocol on Substances that Deplete the Ozone Layer identified methyl bromide as a greenhouse gas of concern (Taylor, 1994; Fields and White, 2002; Arthur, 2008a). The protocol called for the use of methyl bromide to be completely phased out by 2015 except for preshipment and quarantine uses, but there is a provision for critical use exemptions while alternatives are being developed (Fields and White, 2002). Potential alternatives to methyl bromide include fumigants such as sulfuryl fluoride and phosphine, heat treatments, and integrated pest management (IPM) programs which include sanitation, structural modification, contact insecticides, and insecticide aerosol applications.

Tribolium castaneum (Herbst), the red flour beetle, and *T. confusum* Jacquelin du Val, the confused flour beetle, are external stored product pests which have been traditionally treated using methyl bromide fumigations. They are found world-wide in flour mills, retail stores, food processing facilities, and warehouses (Toews et al., 2005). Food industry survey participants identified *Tribolium* spp. as one of their major insect pest problems (Mullen, 2004). In the United States, *T. castaneum* and *T. confusum* are the most abundant and damaging stored product pests in flour mills (Zettler, 1991). They can be found together in the same flour mill (Zettler, 1991) but *T. castaneum* is the

most abundant insect pest captured all year round in flour mills (Campbell and Arbogast, 2004; Toews et al., 2006). *T. castaneum* infests a variety of commodities (Good, 1936) including materials inside milling equipment (Campbell and Arbogast, 2004) and product contamination can result from cast skins, whole adult and immature insects, eggs, dead insect fragments, benzoquinones, and frass (Baur, 1984).

T. castaneum and *T. confusum* frequently have different susceptibilities to insecticides. For example, the LD_{50} for *T. castaneum* (44 µg insecticide/ g of insect weight) is higher than the LD_{50} for *T. confusum* (25 µg insecticide/ g of insect weight) when they are treated with synergized pyrethrin. However, when they are treated with resmethrin the LD_{50} is higher for *T. confusum* (281 µg insecticide/ g of insect weight) than for *T. castaneum* (119 µg insecticide/ g of insect weight) (Zettler, 1991). Differences in efficacy between species have also been reported for diatomaceous earth (Arthur, 2000), hydroprene (Arthur, 2001; Arthur, 2004; Arthur and Hoernemann, 2004), chorfenapyr (Arthur, 2008b), and methoprene and novoluron (Arthur and Fontenot, 2012). The fact that *T. castaneum* and *T. confusum* can exploit hidden areas such as inside building walls, equipment machinery, and cracks and crevices (Campbell et al., 2004) means that a proportion of the population may be able to avoid exposure to insecticides or receive a reduced dose which can increase the difficulty in successfully managing these two insect pest species.

Aerosol insecticides are currently being used in flour mills as part of IPM programs. Aerosol applicationss are defined as the dispersal of insecticides as particles that range in size from 5-50 µm. Commercial applications can be made using a permanently pressurized systems, gas cylinders, or a hand-held applicator (Peckman

and Arthur, 2006). The droplets treat exposed surfaces but cannot penetrate into commodities (Peckman and Arthur, 2006; Arthur, 2008a). Aerosol applications often include a mixture of insecticides such as synergized pyrethrin and methoprene which have different modes of action.

Synergized pyrethrin, a mixture of natural pyrethrins plus a synergist, has been used for the control of stored product pests (Arthur, 2008a). Pyrethrin insecticides are toxic to insects but have a relatively low toxicity to mammals (Hays, 1982; Weinzierl, 1998). They are generally unstable in sunlight, oxygen, or under moist conditions (Barthel, 1973; Weinzierl, 1998). Therefore, synergized pyrethrin tends to cause immediate knockdown of exposed individuals which may result in mortality (Jenson et al., 2010). Adults that are knocked down may not feed or reproduce and could be removed from the facility through sanitation practices.

Methoprene, an insect growth regulator (IGR), is also approved for use in aerosol applications in food processing facilities (Arthur, 2008a). Methoprene is a juvenile hormone agonist which disrupts normal insect development and can cause mortality. In addition to mortality, supernumerary larval molts or intermediate, larval-pupal or pupal-adult, deformities, can occur and these individuals are typically non viable (Beckage, 1998; Henrick, 2007). Furthermore, development on methoprene treated flour can cause *T. castaneum* to develop into sterilized adults (Amos et al., 1978) and increased developmental time of *T. confusum* (Loschiavo, 1975). Both of these sublethal effects can result in a change in population dynamics due to a reduction in the number of offspring produced (Mondal and Parween, 2001). Despite methoprene's broad efficacy against a large number of insect orders it has a short persistence in direct sunlight

(Henrick, 2007). However, when methoprene is applied in-doors it is more persistent in the environment and can be translocated from one surface to another (Henrick, 2007). This results in the redistribution of methoprene and potentially increases its efficacy (Henrick, 2007).

Arthur (2008a) evaluated the effects of a synergized pyrethrin aerosol application in a food warehouse on exposed *T. castaneum* and *T. confusum* adults, pupae, and late-stage larvae. Results showed that *T. castaneum* adults were more susceptible to the synergized pyrethrin aerosol than *T. confusum* adults. Additionally, none of the aerosol exposed late-stage larvae of either species were able to emerge as adults. This finding suggests that the use of synergized pyrethrin alone can control these two beetle species, but the efficacy of synergized pyrethrin has not been evaluated for their eggs. Effects of methoprene applied as an aerosol application in a food warehouse on 3rd and 4th stage *T. confusum* larvae was also explored. Adult emergence was found to be less than 5% for both developmental stages. This finding provides some evidence that use of methoprene as an aerosol application can be effective. However the efficacy of methoprene directly applied as an aerosol has not been evaluated for efficacy to eggs and pupae as an aerosol has not been determined for both *T. castaneum* and *T. confusum*.

Some flour mill pest managers are using aerosol applications of a mixture of methoprene and synergized pyrethrin insecticides in response to the reduction of methyl bromide fumigations (Campbell and Arbogast, 2004). The use of this mixture may be more effective when used together than when used individually since they have different modes of action. Additionally, this approach could help reduce the development of

cross-insecticide resistance. The combination of methoprene and synergized pyrethrin when applied as an aerosol application have been shown to be effective against the stored product pest *Plodia interpunctella* (Hübner), the Indian meal moth (Jenson et al., 2010). This combination has also been shown to provide residual efficacy against *T. castaneum* and *T. confusum* larvae (Sutton et al., 2011). Considering the facts that aerosols primarily contact exposed areas (Peckman and Arthur, 2006; Arthur, 2008a) and that the majority of *Tribolium* spp. populations are typically located in hidden areas (Campbell et al., 2004) it does not initially appear that aerosol applications would be an effective pest management strategy. However, a facility where these aerosol applications are regularly applied as part of an IPM program have shown reduced trap-capture numbers and less fluctuation in abundance of *T. castaneum* (Campbell et al., 2010ab).

The effectiveness of aerosol applications could be enhanced by movement of treated insects or materials such as flour into hidden areas where the majority of the pest population is located. While individual effects of this insecticide translocation may be small the cumulative effects of multiple aerosol applications may contribute to suppressing pest populations. Information on the ability of insecticides to be translocated within a stored product environment is not available. One mechanism which could explain the some of the observed effectiveness of the methoprene and synergized pyrethrin aerosol applications is the horizontal transfer of methoprene.

Horizontal transfer of insecticides occurs when individuals come into contact or ingest an insecticide and move to an area where other insects may come in contact with the insecticide and be affected (Buczkowski and Schal, 2001). Horizontal transfer of

insecticides has been observed in a range of insect systems. For example horizontal transfer of fipronil by *Blattella germanica* (L.), German cockroach (Buczkowski et al., 2001; Buczkowski and Schal, 2001); fipronil by *Blatta orientalis* (L.), Oriental cockroach (LePatourel, 1999; LePatourel, 2000); cypermethrin by *Blatta orientalis* (LePatourel, 1998),S-Methoprene bait by *Solenopsis invicta* (Buren), red imported fire ant (Aubuchon et al., 2006); and pyriproxyfen by *Aedes albopictus* (Skuse) and *Ochlerotatus triseriatus* (Say), mosquitoes (Chism and Apperson, 2003).

In the flour mill system horizontal transfer of insecticides could occur as insects move from areas treated with insecticides into areas that have not been treated or during routine sanitation practices move dead or knocked down individuals and treated flour into hidden areas. In both cases there is the potential for translocation of insecticides but it is unclear to what extent this is occurring and the effect of any translocation on pest populations. Although, it may be possible to horizontally transfer synergized pyrethrin its high insect toxicity (Hays, 1982; Weinzierl, 1998) and knockdown effects (Jenson et al., 2010) do not make it a likely candidate. However, methoprene has been previously been shown to be translocated in other systems (Henrick, 2007) and is more likely to be carried by treated insects into hidden areas since it does not kill adult insects, which are more likely to be directly treated or move across treated areas. This makes methoprene a more likely candidate for horizontal transfer in food facilities.

Tribolium castaneum and *T. confusum* have three traits which could enhance the horizontal transfer of methoprene. These behaviors are movement into and out of hidden resource patches (Campbell and Hagstrum, 2002; Campbell and Runnion,

2003), an aggregation pheromone produced by males which attracts both males and females (Suzuki, 1980; Suzuki, 1981) which could lead to increased encounters among individuals, and cannibalization or necrophagy (Park et al., 1965) which could increase direct contact with treated individuals. These three traits allow for multiple scenarios in which treated individuals could transfer methoprene to another individual. The most likely scenarios or mechanisms are contact with treated individuals, contact and/or ingestion of contaminated substrate, and/or cannibalization of treated individuals.

Horizontal transfer of methoprene by contact occurs when there is direct physical contact between a treated and untreated individual which results in death or other deleterious effects to the secondarily exposed individual. Substrate contamination occurs when a treated individual comes in contact with a material such as flour and some of the methoprene migrates from the cuticle to the flour as the insect moves through the flour. Untreated individuals come in contact with the flour and through cuticular contact with the contaminated flour and/or ingestion of the flour become secondarily exposed. The last potential mechanism for horizontal transfer of methoprene is cannibalization and/or necrophagia. This occurs when a treated individual dies and is consumed by a previously unexposed individual. The *Tribolium* spp. behaviors and the modes of horizontal transfer of methoprene have considerable overlap between each other; this increases the likelihood that horizontal transfer of methoprene may occur in *Tribolium* spp.

While showing evidence for horizontal transfer and its mechanisms is novel for the stored product environment it is not information which is easily transferred to IPM practitioners or related to the overall effects on flour mill insect pest populations.

Additionally, laboratory efficacy studies focused may not necessarily translate to population level responses (Stark and Banks, 2003). Determining pest population response to IPM strategies, under real world conditions, can be hindered by difficulties in sampling populations and replicating studies.

However, population studies can provide a more complete picture of the efficacy of an insecticide since the cumulative effects of both aerosol treatments and horizontal transfer can be evaluated. There are several concerns in looking at population dynamics of *Tribolium* spp. in flour mills. One of them is that most mill pest managers are not willing to have *Tribolium* spp. released into their facility which means that evaluation under less realistic conditions is necessary. Laboratory testing will be difficult since mill managers frequently use multiple tactics such as sanitation, fumigation, aerosol applications, and/or crack and crevice insecticide applications to control *Tribolium* spp. These IPM tactics are difficult to evaluate and/or replicate.

Additionally, there is limited data on pest population dynamics and their response to these management strategies (Campbell et al., 2002). The information currently available for the effects of treatments on *T. castaneum* populations and populations is focused on the effect of structural treatments such as fumigations of methyl bromide and/or sulfuryl fluoride (Campbell et al., 2010ab) and heat treatments (Mahroof et al., 2005) The effects of other treatments in food facilities, such as aerosol applications of insecticides, surface or crack and crevice insecticide treatments, and/or sanitation measures have not been as well documented. The addition of aerosol applications, spot-insecticide treatments, and/or sanitation in combination appear to reduce the rate of rebound of *T. castaneum* after fumigation (Campbell and Arbogast, 2004) but it is not

possible to isolate the role of the aerosol insecticides. Information on the different components of aerosol applications, individual and in combination, on small populations, large populations with sanitation and refuge areas, and large populations with refuge areas can provide useful data as to how the population is responding to the aerosol applications.

Determining the potential for horizontal transfer, the mechanisms behind the transfer and the effects of horizontal transfer on population growth provide insight into the potential of aerosol applications to effect *T. castaneum* and *T. confusum* populations within a flour mill. Horizontal transfer in this system offers a unique and novel mechanism for potentially controlling pest populations. This is especially important when one considers the increased costs of insecticide development and registration along with improved knowledge regarding the effect of insecticide use on the environment. Considering the population effects on *Tribolium* spp. increases the breadth of knowledge regarding *Tribolium* spp. This information could be relayed to an insect pest manager in a mill and provide greater confidence in the effects of IPM measures already in use. This research should help answer many questions regarding *Tribolium* spp. and the effect of methoprene and synergized pyrethrin aerosol applications.

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Chapter 2 - Horizontal Transfer of Methoprene by *Tribolium castaneum* (Herbst) and *T. confusum* Jacquelin du Val

Abstract

Experiments were performed to evaluate the potential for horizontal transfer of methoprene from *Tribolium castaneum* (Herbst) or *T. confusum* Jacquelin du Val treated with the insect growth regulator methoprene to other individuals of the same species that were not treated (i.e., secondary exposure). In the first experiment, late-stage larvae that were secondarily exposed to methoprene had a lower percentage of adults without external deformities that survived to the end of the observation period (72%) than those exposed to control treatments (90%). *T. castaneum* was more susceptible to the effects of horizontal transfer, with 74% survival compared to 90% for *T. confusum*. In the second experiment, early-stage larvae, late-stage larvae, and pupae were found to have the same susceptibility to horizontal transfer of methoprene. In the final experiment, the potential for sub-lethal effects resulting from secondary exposure of *T. castaneum* late-stage larvae to methoprene was assessed, but survival to adulthood was not affected by the methoprene treatment and there were no differences in the number of eggs laid by normal appearing adults exposed to methoprene during development. These results indicate that horizontal transfer may occur with these species and contribute to insecticide efficacy in food facilities, but effects may be limited and variable.

Introduction

Aerosol insecticides are used as part of integrated pest management (IPM) programs in food processing and storage facilities. Aerosol applications, i.e., fogging or

ultra-low volume (ULV), involve applying an insecticide in small droplets (5-15 µm) under pressure using either a portable applicator or permanently installed system (Peckman and Arthur, 2006). Aerosol insecticide formulations, unlike fumigants, are not able to penetrate into products or obstructed areas (Peckman and Arthur, 2006; Arthur, 2008). Therefore, aerosol insecticide applications are predicted to cause direct mortality only to individuals in open areas that are directly exposed to settling droplets or encounter residual insecticide on treated surfaces. However, because most of a pest population, especially the immature stages, occurs in hidden harborages or refugia where food accumulates (e.g., wall voids, cracks, and equipment) it is challenging to facilitate direct exposure of the insects to the insecticide (Campbell et al., 2004).

Aerosol insecticide formulations used in the food industry typically use a combination of pyrethroid or synergized pyrethrins, for immediate knock-down and mortality, and an insect growth regulator (IGR) such as methoprene, which can negatively effect immature development. Methoprene is an analog of juvenile hormone that can delay and/or halt immature development and causing physical deformities during molting, but has little effect directly on adults (Henrick, 2007). Individuals exposed as immatures to an IGR such as methoprene can have reduced survival to adult stage (Loschiavo, 1974; Williams and Amos, 1974; Arthur, 2001; Arthur and Hoernemann, 2004) and reduce fertility (Loschiavo, 1974; Oberlander et al., 1997; Chanbang et al., 2007). These effects have the potential of effecting the population growth in insect populations (Mondal and Parween, 2001).

Tribolium castaneum (Herbst) (red flour beetle) and *T. confusum* Jacquelin du Val (confused flour beetle) are stored-product pests frequently found worldwide in post-

harvest facilities, particularly flour mills. They are often less susceptible to insecticides compared to other stored-product beetles, but the two species can differ in their relative susceptibility to a specific insecticide (Zettler, 1991; Arthur, 2001; Arthur and Hoernemann, 2004; Arthur and Fontenot, 2012). Additionally, these species often exploit hidden areas inside a post-harvest facility, which makes them difficult to treat with insecticides (Campbell et al., 2004; Toews et al., 2005). The ability of fumigants to penetrate into these hidden areas made them effective at managing this pest (Campbell et al., 2010ab), but with decreasing use of fumigation and increasing use of aerosols this lack of penetration could result in less effective IPM programs. However, a mixture of synergized pyrethrin and methoprene aerosol treatments as part of an IPM program that included sanitation tended to reduce large fluctuations in *Tribolium castaneum* populations within a mill (Campbell et al., 2010ab). The specific mechanism for its effectiveness has not been determined.

Inside post-harvest facilities it may be possible for insecticides to be translocated, transported, into hidden areas where populations of *Tribolium* spp. occur by movement of materials or insects that have come in contact with aerosol droplets or residual insecticide on surfaces. Horizontal transfer of insecticides occurs when an individual comes in contact with an insecticide, returns to an aggregation site, and other individuals come in contact with the insecticide and suffer from deleterious effects such as death (Buczkowski and Schal, 2001). Horizontal transfer of insecticides using a variety of mechanisms has been observed in a range of insects: e.g., the Oriental cockroach, *Blatta orientalis* (L.) (LePatourel, 1998; LePatourel,1999; LePatourel, 2000); the German cockroach, *Blattella germanica* (L.) (Buckzkowski et al., 2001; Buczkowski

and Schal, 2001); mosquitoes, *Aedes albopictus* (Skuse) and *Ochlerotatus triseriatus* (Say) (Chism and Apperson, 2003); and the red imported fire ant, *Solenopsis invicta* (Buren) (Aubuchon et al., 2006). Insect growth regulators such as methoprene are good candidates for horizontal transfer for several reasons. They do not cause mortality of adult stages (Strong and Diekman, 1973; Mondal and Parween, 2000), which enables dispersing adults to pick up the insecticide and transport it into hidden areas occupied by immature stages. Translocation, movement of insecticides, between surfaces is likely to occur under inside conditions (Henrick, 2007). Furthermore, IGRs are often effective at low concentrations (Loschiavo, 1974; Hahn et al., 2001) and insects which are secondarily exposed are more likely to receive a small amount of the original insecticide.

Horizontal transfer of methoprene is predicted to occur with *Tribolium* spp., most likely between adults and immatures, for several reasons. First, adult *Tribolium* frequently move among flour patches and thus are more likely to be exposed directly to the insecticide treatment or to treated surfaces (Campbell and Hagstrum, 2002; Campbell and Runnion, 2003). Second, males produce an aggregation pheromone, which increases the likelihood of finding multiple individuals in the same location (Suzuki, 1980; Suzuki, 1981). These two aspects of their behavior increase the likelihood of dispersing individuals being exposed to methoprene and then moving into locations where other non-dispersing individuals are exposed by either direct contact or by transfer through the food substrate. Third, *Tribolium* spp. adults and larvae are cannibalistic and can feed on living eggs, larvae, pupae and newly emerged adults (Park et al., 1965) and also on dead individuals. Cannibalism may increase horizontal

transfer through increased physical contract with treated insects or consumption of insecticide. These behavioral and biological phenomena allow for three potential methods of horizontal transfer of methoprene in *Tribolium* spp.: physical contact with treated individuals, contact with contaminated substrate i.e. food source, and cannibalization by predation or scavenging of treated individuals.

In this series of experiments, the potential for horizontal transfer of methoprene to occur was evaluated for *T. castaneum* and *T. confusum*. The effects on survival and development of immature developmental stages after exposure to methoprene treated developmental stages were assessed by determining the length of survival time and the normal, adult survival. Length of survival time is the number of days that focal insects were alive after exposure to treatments. Normal adult survival was determined by the adults that did not have external deformities and were alive at the end of the observation period. The potential for delayed effects was also evaluated by measuring oviposition by morphologically normal females that had been exposed to methoprene treated individuals during development.

Materials and Methods

Laboratory strains of *T. castaneum* and T. *confusum*, both originally collected in Kansas prior to 1958, were used in experiments. Laboratory cultures were maintained at 28.34±0.02^{o}C, 46.26±0.08% RH, and 14/10 (day/night) photoperiod in an incubator (I-36 Series Incubator, Percival Scientific Inc., Perry, IA, USA). Insects were cultured by adding 100 adults to a 280 g mixture of unbleached wheat flour (Stafford County Flour Mills Co., Hudson, KS, USA) and brewer's yeast (MP Biomedicals LLC, Solon, OH, USA), 5% by weight, in a 720 ml glass canning jar (Jarden Home Brands, Daleville, IN,

USA). Three days later, adults were removed by sieving with a size 30, 0.59 mm (diameter of the mesh sieve openings), mesh sieve (W. S. Tyler Co., Mentor, OH, USA), the flour was returned to the jar, and the jar was returned to culture conditions described above until the next generation of adults emerged. The insects used in experiments were collected a month after the culture jars were first inoculated with adults. Voucher specimens were deposited at the Kansas State University Museum of Entomological and Prairie Arthropod Research (KSU-MEPAR) under voucher number 226.

Does horizontal transfer of methoprene occur between treated individuals and untreated larvae?

Thirty late stage larvae, pupae, or adults were placed in concrete exposure dishes consisting of a plastic Petri dish bottom (62 cm^2 surface area) filled to a depth of 0.5 cm with concrete patching material (Rockkite®, Hartline Products, Co., Inc., Cleveland, OH, USA). The procedure for creating these concrete exposure arenas has been previously described in detail (Arthur, 2008).

The labeled rate of methoprene (Diacon® II, Central Sciences International, West Schaumberg, IL, USA) for an aqueous spray treatment (1 ml methoprene/3785 ml water/10,763 cm^2 surface area treated) was applied using an artist's airbrush (Badger Air-Brush Company, Franklin Park, IL, USA) to treatment dishes to simulate an aerosol application. This rate converted to 0.0192 mg of active ingredient in deionized water for a total volume of 0.25 ml applied to each dish. Each water control Petri dish received 0.25 ml of deionized water.

Treatment dishes and insects were allowed to dry uncovered at ambient room temperature for approximately 15 min before insects were transferred to new Petri

dishes. Insects were then frozen to prevent degradation of methoprene (Henrick, 2007) and to standardize conditions among treatments by placing the dishes in the freezer section of a Hotpoint household refrigerator/freezer (General Electric Co., Louisville, KY, USA) for 24 hrs at 54.0±0.2% RH and -16.8±0.0^{o}C.

Focal individuals (i.e., untreated larvae not directly sprayed with insecticide or water) were placed in 17.7 ml (7.9 cm long X 1.7 cm wide) polystyrene shell vials (Daigger, Vernon Hills, IL, USA) containing 1.1±0.0 g of flour. Immediately after adding focal individuals to vials, a thawed (at ambient room temperature for approximately 10 mim) dead larva, pupa or adult treated with either methoprene or water was placed in the vials. An untreated control group consisted of just flour and focal larva in the vial, but no treated developmental stage. Vials were held in an incubator (model 3710 S/N 34798-32, FormaScientific Inc., Marietta, OH, USA) at 26.5±0.0^{o}C and 79.4±0.1% RH.

Focal individuals were observed daily for 15 to 30 days and the following information recorded: stage of development (larva, pupa, or adult), whether individual was alive or dead, and if external physical deformities were observed. The level of cannibalization on the treated developmental stages was evaluated by observing the developmental stages with a microscope (Wild Heerbrugg Plan 1X, Leico Geosystems, Canton St. Gallen, CH) and quantifying the cannibalization using an index where 0 = no damage; 1 = one or two bites in the body and/or scratches on the cuticle signifying attempts at feeding; 2 = missing appendages and/or part of the abdomen was damaged signifying some feeding but not a significant amount; and 3 = complete consumption.

The experiment was blocked by day, with seven blocks performed with 3 to 11 replicates per treatment combination (*T. castaneum* or *T. confusum* focal insects under

control or flour plus a water or methoprene treated developmental stage treatment conditions) per block. This resulted in a total of 37 to 39 focal individuals (replicates) per treatment combination.

Statistical analysis was performed on two types of data. The first type of data was the day that mortality of focal insects occurred during the experimental period. This type of data provides an indicator of the effect of the level of secondary methoprene exposure since it can detect differences in when mortality is observed. This data was analyzed using a Kaplan-Meir log-rank survival analysis test and the Holm-Sidak method for pair-wise comparisons when significant differences are found between more than two pairs (SigmaPlot v. 11.0 software, Systat Software Inc., Chicago, IL, USA).

The second type of data was the number of focal insects without external deformities that were alive at the end of the observation period, normal surviving adults, was compared among treatment groups. This response variable encompassed the observations for mortality, deformities, and adult emergence and provided an overall measure of the effects of the treatment. Because individual insects were considered replicates and the response was binary (yes/no) this generated non-normal binary datasets. Therefore, this data was analyzed using the GLIMMIX procedure (SAS software v. 9.2, SAS Institute, Cary, NC, USA). Each analysis was evaluated for random effects, caused by the block, individual (replicate), or block by individual interactions, and the appropriate random statements applied to each analysis. In cases where the covariance parameters in the random statements all equaled zero the random statement was removed from the model. Tukey–Kramer grouping for least square means, within the GLIMMIX procedure, was used as a means separation test.

Does developmental stage of individual influence susceptibility to horizontal transfer of methoprene?

T. castaneum and *T. confusum* late-stage larvae and pupae were collected, treated, and added to vials as described above. The focal insects used in the experiment were larvae collected from jars established 4 weeks prior (a mixture of 3rd and 4th stage larvae (early-stage larvae)), larvae collected from jars established 5 weeks prior (5th stage larvae (late-stage larvae)), and pupae. One focal insect (early stage larva, late stage larva, or pupa) was placed in a vial with either 1 g of flour only (untreated control), 1 g of flour and a water treated late-stage larva or pupa, or 1 g of flour and a methoprene treated late-stage larva or pupa. Five replications of each treatment combination were performed within a block, day, and the procedure was repeated three times for a total of 15 replications per treatment combination. The focal insects were observed weekly for three weeks and the deformities, adult emergence, and mortality was recorded. GLIMMIX procedure was used to analyze the number of normal surviving adults to the end of the observation period. To evaluate the simplest model for the normal, surviving adults for each species the developmental stage for all treatments was evaluated first and then each treatment with the different developmental stages; the species were compared to each other across the treatments. Because of the reduced number of observations, time of mortality was not analyzed.

Do adults without external deformities after secondary exposure to methoprene exhibit delayed effects on reproduction?

The first part of this experiment was conducted to generate normal surviving adults after exposure to methoprene treated individuals that could be evaluated for the effect on reproduction. *T. castaneum* adults were collected, treated with methoprene,

and processed as described above. One focal individual (late-stage larva), collected as described above, was placed in a polystyrene shell vial (11.0 ml, 5.2 cm long x 1.6 cm wide) with 1 g of flour (untreated control) or with 1 g of flour and one dead methoprene treated adult (methoprene treated). Methoprene treated and untreated control combinations each had 100 vials within each block, day, and two blocks were performed. The focal insects were held for 15 days and observed daily for survival, presence of deformities, and adult emergence. Normal appearing pupae were sexed by observing the genital papillae (Halstead, 1963) under a microscope. The resulting normal surviving adults' data was analyzed as described above to determine the effect of secondary exposure to treated adults.

Normal surviving adults that were between 4 and 8 days post adult emergence were paired together. Amos et al. (1978) found that crosses between deformed *T. castaneum* were not viable so these individuals were not evaluated in the current study. Pairings consisted of control male and control female (control individuals had been exposed to flour only) (CM X CF), control male and methoprene exposed female (methoprene exposed individuals were held in flour with a methoprene treated adult during development) (CM X MF), methoprene exposed male and control female (MM X CF), and methoprene exposed male and methoprene exposed female (MM X MF). The individuals were assigned to pairs using a random number table. Number of pairs used per combination depended on the number of normal surviving adults in the different groups: there were 19-23 pairs for each treatment combination.

The pairs were placed in new vials with 1 g of flour, which had been sieved twice through a size 60, 0.25 mm, mesh sieve (W.S. Tyler Co., Mentor, OH, USA) to facilitate

the separation of eggs from the flour later in the experiment. Pairs were transferred to a new vial every two days for two weeks. The flour in vials after adult removal was passed through a size 50, 0.30 mm mesh sieve (W.S. Tyler Co., Mentor, OH, USA) to remove the eggs and the number of eggs was counted and recorded. Pairings with both individuals alive at the end of the two weeks were considered successful replicates. To determine differences in total number of eggs among treatments, data was analyzed using a general linear models procedure (SAS software v. 9.2, SAS Institute, Cary, NC, USA). A repeated measures test was also performed on the number eggs oviposited over time using the GLIMMIX procedure (SAS software v. 9.2, SAS Institute, Cary, NC, USA).

Results

Does horizontal transfer of methoprene occur between treated individuals and untreated larvae?

The rate of mortality was simplified by comparing the controls and methoprene treatments independently for each species. The *T. castaneum* control treatments (flour only or flour and a water treated late-stage larva, pupa, or adult) were not different from each other ($Z=0.207$, d.f.=3, $P=0.976$) and were combined for further analysis. The methoprene treatments (flour and a methoprene treated late-stage larva, pupa, or adult) were also not different ($Z=2.146$, d.f.=2, $P=0.342$) from each other and combined for further analysis. Analysis comparing the combined controls to the combined methoprene treatments showed a significantly higher rate of morality for focal insects

that were exposed to methoprene treated developmental stages than those exposed to control treatments (Z =9.529, d.f.=1, P=0.002).

T. confusum rate of mortality in control treatments was not significantly different (Z=2.401, d.f.=3, P=0.493), and data for the control treatments were combined for further analysis. Mortality rate in the methoprene treatments was also not significantly different (Z=2.546, d.f.=2, P=0.280) and were combined for further analysis. Analysis comparing the combined controls and combined methoprene treatments showed that they were not significantly different (Z=0.113, d.f.=1, P=0.737). The analysis comparing *T. castaneum* and *T. confusum* and the combined controls and methoprene treatments showed that *T. castaneum* had a significantly greater rate of mortality compared to *T. confusum* (Z=23.146, d.f.=3, P<0.001) (Fig. 2.1).

The normal surviving adult data was simplified in a similar way. *T. castaneum* normal surviving adults from control treatments (flour only and flour and a water treated larvae, pupae, and adults) were not significantly different from each other (F=0.05; d.f.=3, 127; P=0.9840), and were combined for further analysis. Focal individuals exposed to methoprene treated adults and methoprene treated larvae were significantly different from each other, but methoprene treated pupae were not different from any other methoprene treatment (F=3.21; d.f.=2, 96; P=0.0448). Therefore, the methoprene treatments were not combined.

Comparing the grouped controls to the methoprene treatments showed *T. castaneum* individuals in the combined controls was significantly greater number of normal surviving adults than the focal individuals exposed to methoprene treated pupae or larvae but the same as those exposed to methoprene treated adults (F=10.08; d.f.=3,

242; P<0.001; Fig. 2.2). Additionally, all of the methoprene treated developmental stages had the same number of normal, surviving adult.

For *T. confusum,* the percentage of normal surviving adults in control treatments (flour only or water treated larva, pupa, or adult) were not significantly different from each other (F=0.41; d.f.=3, 137; P=0.7479), therefore they were combined for further analysis. The methoprene treatments were not evaluated independently in order to maintain the treatment analysis structure for comparison between the two species. The *T. confusum* exposed to the combined control conditions were not significantly different from the individuals exposed to methoprene treated adults but were significantly different from individuals exposed to methoprene treated larvae or adults (F=5.77; d.f.=3, 256; P<0.0008) (Fig. 2.2). The methoprene developmental stages were not different from each other.

Analysis using the two species and comparing the combined controls to the methoprene treatments (flour and methoprene treated larva, pupa, or adult) found that the number of normal surviving adults was significantly greater for *T. confusum* than for *T. castaneum* (F=13.98; d.f.=1, 515; P=0.0002) and significantly less for individuals that were exposed to methoprene treated developmental stages (F=13.71; d.f.=3, 515; P<0.0001), but there was no species by treatment interaction (F=1.11; d.f.=3, 515; P=0.3462).

The level of cannibalization (Table 2.1) was relatively low for both *Tribolium* species and in control and methoprene exposed treatments. Additionally, there were a low number of individuals that suffered deleterious effects (i.e., mortality or external deformities) at each cannibalization ranking (Table 2.1). In order to determine if feeding

on a water or methoprene treated developmental stage resulted in deleterious effects, the data for developmental stages (late-stage larvae, pupae, and adults) treated with water were combined and data for those stages treated with methoprene were combined. *T. castaneum* late-stage larvae that were exposed to the combined methoprene treated developmental stages (late-stage larvae, pupae, and adults) had a significantly greater (F=8.18; d.f.=1, 213; P=0.0046) percentage of individuals with deleterious effects than those in the combined controls (flour only or water treated larvae, pupae, and adults). The level of cannibalization (rank) did not significantly affect the number of individuals with deleterious effects (F=22; d.f.=3, 213; P=0.8847) and the interaction between the treatment and level of cannibalization was also not significant (F=0.02; d.f.=3, 213; P=0.9968).

T. confusum focal insects did not feed at the rank 2 level in either the control or methoprene groups but to maintain the treatment analysis structure for analysis between the two species ranks 2 and 3 were combined. For *T. confusum,* individuals exposed to the combined methoprene treated developmental stages had significantly greater (F=8.98; d.f.=1, 182; P=0.0031) percentage of deleterious effects compared to individuals in the combined controls (Table 1) but the level of cannibalization was not different between the treatments (F=2.15; d.f.=2, 182; P=0.1192) nor was there an interaction between the treatment and the cannibalization level (F=0.05; d.f.=2, 182; P=0.9556).

In order to compare cannibalization between the two species, the ranks 2 and 3 were combined for further analysis. The two species did not differ in deleterious effects (F=2.40; d.f.=1, 438; P=0.1220), nor in the level of cannibalization (rank) (F=1.76;

d.f.=2, 438; P=0.1734). However, the percentage of individuals with deleterious effects resulting from exposure to methoprene treated developmental stages was significantly higher (F=21.27; d.f.=1, 438; P<0.0001) than the percentage of individuals with deleterious effects after exposure to water treated developmental stages. None of the interactions between the treatment, level of cannibalization, and species was significantly different: treatment by rank (F=0.04; d.f.=2, 438; P=0.9576), treatment by species (F=0.34; d.f.=1, 438; P=0.5581), species by cannibalization rank (F=1.35; d.f.=2, 438; P=0.2600), and treatment by rank and by species (F=0.06; d.f.=2, 438; P=0.9430).

Does developmental stage of individual influence susceptibility to horizontal transfer of methoprene?

The *T. castaneum* pupae, early-stage larvae, and late-stage larvae that were exposed to a methoprene treated developmental stage had a significantly lower percentage of normal surviving adults without deformities (F=4.57; d.f.=2, 70; P=0.0136, F=4.61; d.f.=2, 72; P=0.0130, F=4.11; d.f.=2, 70; P=0.0205, respectively) than focal insects that were exposed to flour only or flour and a water treated developmental stage (Fig. 2.3a). Each treatment (combined controls, flour and methoprene treated late-stage larva, and methoprene treated pupa) was evaluated separately to determine differences between developmental stages (early-stage larvae, late-stage larvae, and pupae) exposed to treatment conditions. The percentage of normal surviving adults was not significantly different for any of the developmental stages within the treatments: combined controls (F=0.07; d.f.=2, 132; P=0.9318), a methoprene treated larva (F=0.00; d.f.=2, 28; P=0.9992), or a methoprene treated pupa (F=0.00; d.f.=2, 28; P=1.0000).

The analysis for normal surviving *T. confusum* adults was evaluated in the same manner. The early-stage larvae and pupae that were exposed to methoprene treated developmental stages (late-stage larvae and pupae) were the same (F=0.18; d.f.=2, 72; P=0.8343, F=0.72; d.f.=2, 72; P=0.4891; respectively) (Fig. 2.3b). The *T. confusum* late-stage larvae that were exposed to methoprene treated developmental stages had a significantly lower (F=3.34; d.f.=2, 72; P=0.0408) percentage of normal surviving adults than the focal insects that were exposed to the control conditions. The individuals exposed to a methoprene treated late-stage larva or pupa were not significantly different from each other. The percentage of normal surviving adults was not significantly different for any of the developmental stages within the treatments: combined controls (F=0.23; d.f.=2, 130; P=0.7966), a methoprene treated larva (F=2.06; d.f.=2, 42; P=0.1399), or a methoprene treated pupa (F=0.36; d.f.=2, 40; P=0.7031).

Analysis evaluating the two species*. T. confusum* and *T. castaneum*, found that the focal insects that were exposed to the combined control (flour only or flour and a water treated larva or pupa) had the same percentage of normal surviving adults (F=0.05; d.f.=1, 262; P=0.8301), the three focal developmental stages (early-stage larvae, late-stage larvae, and pupae) were not significantly different (F=0.15; d.f.=2, 262; P=0.8635), and the interaction between the stage and the species was the same (F=0.15; d.f=.2 ,262; P=0.8634). Focal insects that were secondarily exposed to a methoprene treated late-stage larva or pupa that were surviving adults without deformities was significantly higher (F=4.03; d.f.=1, 70; P=0.0487, F=7.07; d.f.=1, 70; P=0.0097, respectively) in *T. confusum* than in *T. castaneum*. There was no significant differences in the focal developmental stages that were secondarily exposed to a

methoprene treated larva (F=1.37; d.f.=2, 70; P=0.2670) or a methoprene treated pupa (F=2.20; d.f.=2, 70; P=0.8220) and there were no significant difference for the interaction between the exposed developmental stage and the two species for focal insects that were exposed to a methoprene treated larva (F=1.41; d.f.=2, 70; P=0.2518) or to a methoprene treated pupa (F=0.21; d.f.=2, 70; P=0.8142).

Do adults without external deformities after secondary exposure to methoprene exhibit delayed affects on reproduction?

The percentage of focal *T. castaneum* that were normal, surviving adults was not significantly different (F=0.63; d.f.=1, 380; P=0.4282) (Fig. 2.4) between the group exposed to a methoprene treated adult in flour and the group exposed to flour only. Overall there was a higher percentage of females (53%) than males (47%) but this was not significantly different (F=1.91; d.f.=1, 370; P=0.1678). Males and females did not differ in the percentage with deformities (39% and 31%, respectively) (F=0.20; d.f.=1, 370; P=0.6544). Evaluating just the presence of external deformities there was also no difference between individuals exposed to a methoprene treated adult in flour (37%) and those exposed to flour only (33%) (F=0.66; d.f.=1, 370; P=0.4179).

When evaluating potential delayed effects of exposure to methoprene treated individuals, there was no difference in the total number of eggs laid among the combinations or methoprene and control exposed males and females (F=0.18; d.f.=3, 521; P=0.9094) (Fig. 2.5). There was also no effect of methoprene exposure on the temporal pattern of oviposition using repeated measures analysis (F=1.17; d.f.=3, 518; P=0.3192). However, there was a significant effect of block (F=5.98; d.f.=1, 28; P=0.0210), time point (day when eggs were oviposited) (F=11.17; d.f.=6, 518;

P=0.0002), and a significant interaction between the two (F=4.46; d.f.=6, 518; P=0.0002). The Tukey-Kramer least square means test found that the second block had a higher number off eggs laid than the first block.

Discussion

The significantly reduced differences survival rates and percentage of normal surviving adults observed in the *Tribolium* species demonstrate that the horizontal transfer of methoprene can occur between treated and untreated immatures under laboratory conditions. *T. castaneum* was more susceptible to the effects of horizontal transfer of methoprene than *T. confusum,* which is consistent with earlier research showing that *T. confusum* tends to be less susceptible to methoprene (Arthur, 2008). Early developmental stage (3^{rd} and 4^{th} stage) larvae, late-stage (5^{th} stage) larvae, and pupae were similar in susceptibility to horizontal transfer of methoprene, even though they were exposed as immatures to methoprene for different lengths of time.

The role of cannibalism in horizontal transfer of methoprene is less clear but does not appear to be a factor. For *T. castaneum*, cannibalism did increase the percentage of individuals showing deleterious effects such as external deformities and mortality. However, the GLIMMIX analysis did not find the level of cannibalization to be a significant factor for the deleterious effects. There was no effect of cannibalism with *T. confusum* which was less susceptible overall to methoprene. This may be due to the relatively low number of individuals feeding at ranks 1, 2, and 3 compared to no feeding (rank 0) which would make it difficult detect significant differences even with high percentageages of deleterious effects. However, individuals secondarily exposed to methoprene were found to be significantly different, regardless of the cannibalization

level, from the control combinations (water treated larvae, pupae, or adults). This suggests that cannibalization, when it occurs, may be less important to horizontal transfer of methoprene but contact with a methoprene treated individuals or flour that has been contaminated with methoprene may be more likely to result in horizontal transfer of methoprene. However, this may be species or system specific as cannibalization has been thought to result in horizontal transfer of insecticides in cockroaches (LePatourel, 1999; Buczkowski and Schal, 2001; Buczkowski et al., 2001)

The 60-80% normal surviving adults after exposure to a methoprene treated developmental stage suggests a weak effect of horizontal transfer of methoprene; at least under the experimental conditions used in these experiments. It is possible that weak cumulative effects of horizontal exposure or additional sublethal effects such as sterilization or reduced fecundity may result in large effects on population growth. Sublethal effects of methoprene exposure have been previously reported in *T. castaneum* (Amos, et al., 1978) .

In the current experiment, there was no evidence for sublethal/delayed effects due to horizontal transfer of methoprene in *T. castaneum*. However, there were no significant effects on the percentage of normal surviving adults due to the methoprene treatment either even though this experiment was similar in design to the first two experiments where significant effects were observed. It is possible that the same number of eggs being oviposited by the females in the different pairing treatment groups (CM X CF, CM X MF, MM X CF, and MM X MF) is real. Amos et al. (1978) also found no delayed effects on oviposition comparing normal *T. castaneum* adult females exposed to a methoprene treated diet from the egg stage and control females.

However, other studies found a reduction in the F1 progeny yield of *T. castaneum* that were exposed to a methoprene treated diet which was initiated in the larval stage (Wijayaratne et al., 2012; Eisa et al., 1984) and the adult stage (Wijayaratne et al., 2012; Mian and Mulla, 1982). It is possible that in the current study if we had extended the experiment to include the evaluation of adult F1 progeny additional effects might have been detected.

In conclusion, the experiments indicate that horizontal transfer of methoprene can occur under lab conditions. The current experiments do not provide evidence for which mechanism is used for transfer. Cannibalization results in both direct and potentially prolonged contact with a treated individual and the consumption of insecticide. In the experiments reported here, there was not a relationship between effects and level of cannibalization but this needs further evaluation. In many food facilities aerosol insecticide applications involve treatment with synergized pyrethrin or pyrethroid insecticides which cause knock down and mortality and this might increase the potential for cannibalization being a mechanism. Contact between individuals alone, without feeding, could also be sufficient for secondary exposure to methoprene if it moves readily between individuals. Substrate contamination may also occur due to the movement of a methoprene treated developmental stage though flour thereby contaminating it so that untreated individuals can become secondarily treated with methoprene. Understanding the effect of horizontal transfer of methoprene and the role of the different mechanisms of transfer could provide a partial explanation for why aerosol treatments appear to be effective in controlling *Tribolium* species populations in flour mills.

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Figures and Tables

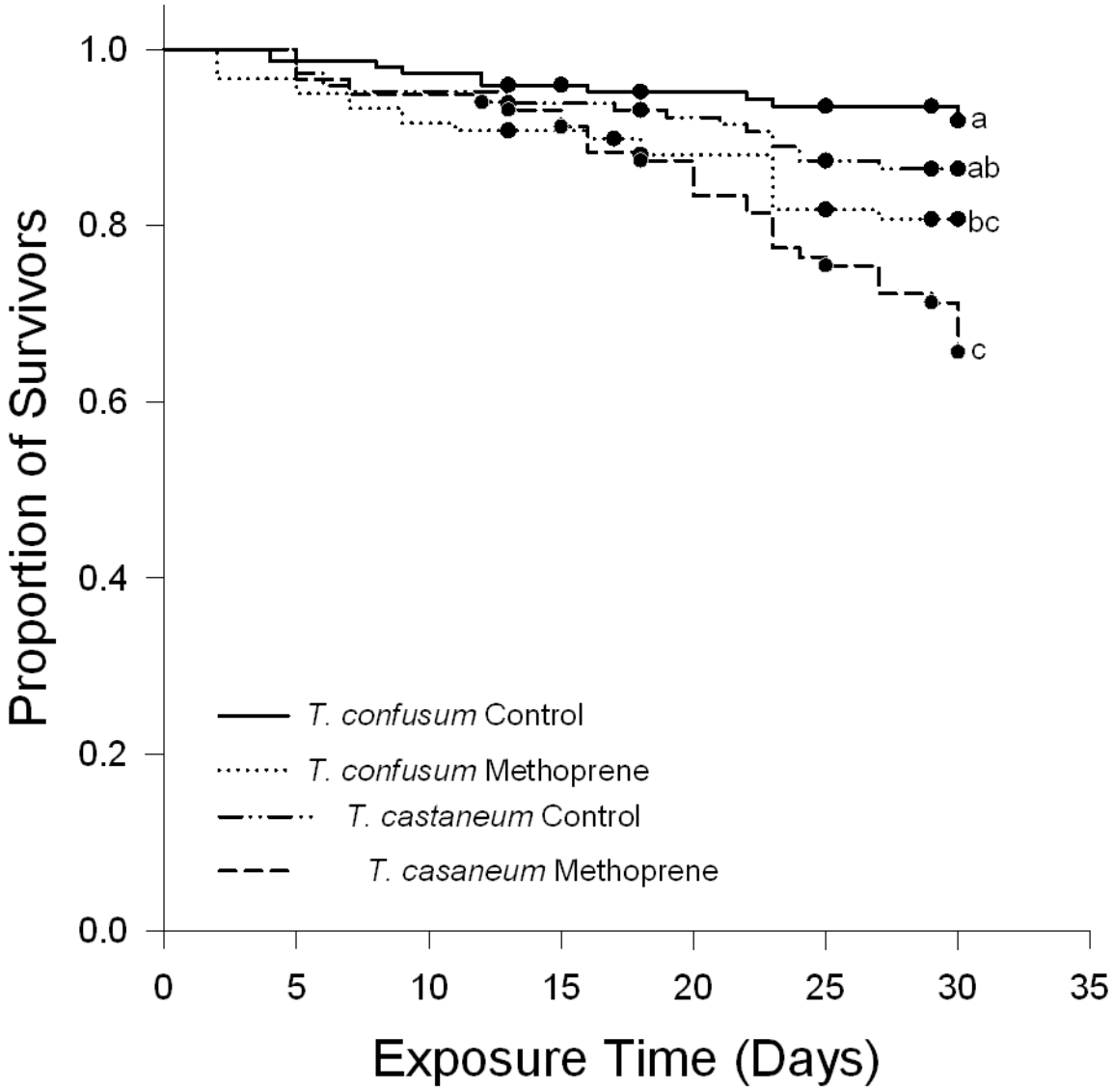

Figure 2.1 The survival curves for *T. castaneum* and *T. confusum* focal insects during exposure a methoprene treated larva, pupa, or adult in flour (Methoprene) to combined control treatments of flour only or flour and a water treated larva, pupa, or adult (Control); with black circles indicating censor points where the experimental block was terminated and lines with the same lower case letters representing responses that are not significantly different according to Holm-Sidak pair-wise comparisons (P>0.05).

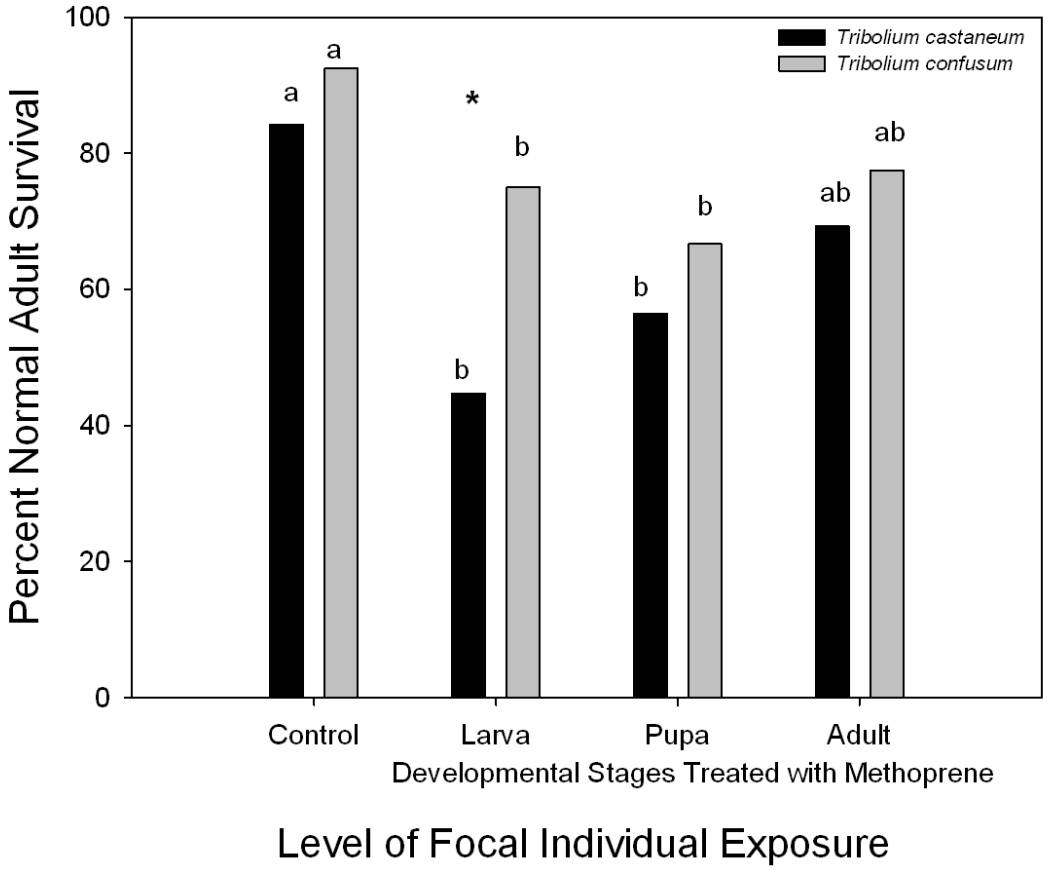

Figure 2.2 The percentage of normal *T. castaneum* and *T. confusum* surviving adults after exposure to a methoprene treated larva, pupa, or adult in flour or a control (combined data from flour alone and water treated larva, pupa or adult in flour). Bars with the same lower case letters, within a species, or an asterisk, between species, have means which are not significantly different from each other according to least square means test with a Tukey-Kramer adjustment for multiple comparisons (P>0.05). Since each focal insect is considered a replicate a true mean or measure of error cannot be generated.

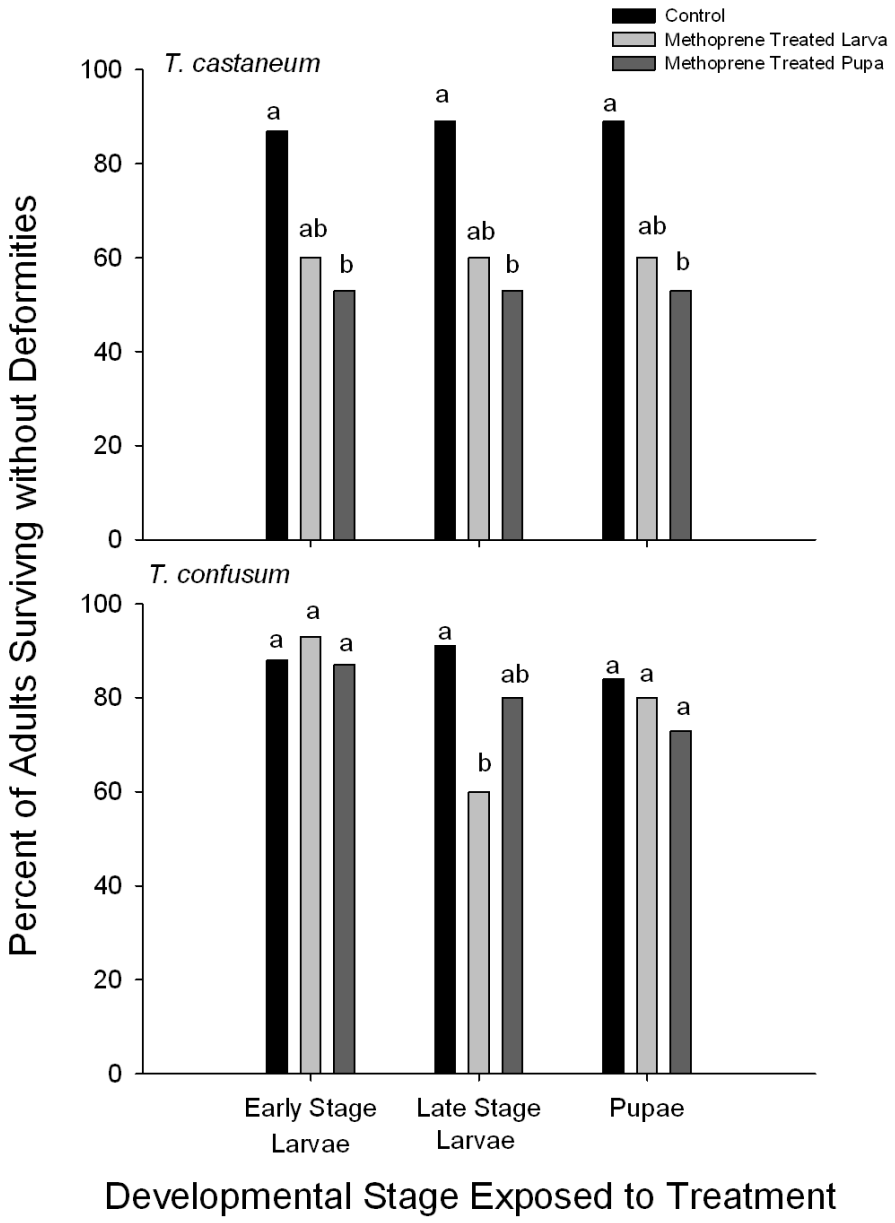

Figure 2.3 The percentage of *T. castaneum* (A) and *T. confusum* (B) early-stage larvae (3rd and 4th stage larvae), late-stage larvae (5th stage larvae), or pupae which had normal surviving adults after exposure to flour only or flour and a water treated larva or a pupa (Control), or exposure to flour and a methoprene treated larva or pupa. Bars with the same lower case letters have treatment means that are not significantly different within a developmental stage according to least square means test with a Tukey-Kramer adjustment for multiple comparisons (P>0.05). Since each focal insect is considered a replicate a true mean or measure of error cannot be generated.

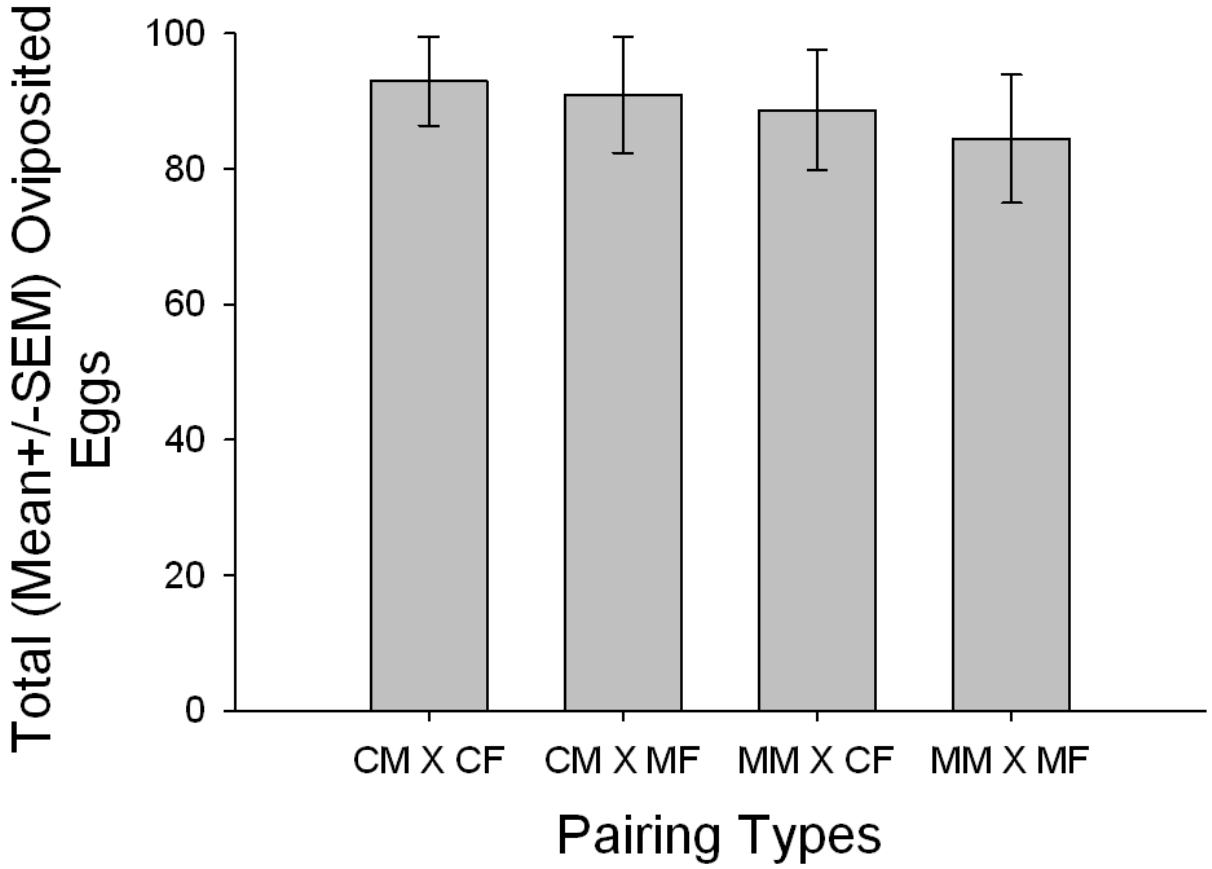

Figure 2.4 The total number (mean ±SEM) of the of eggs laid by different pairs of *T. castaneum* adults that were exposed as immatures to flour only (CM- control male, CF- control female) or flour and a methoprene treated adult (MF- methoprene male, MF- methoprene female); there were no significant differences between treatments according to least square means test with a Tukey-Kramer multiple comparisons adjustment (P>0.05).

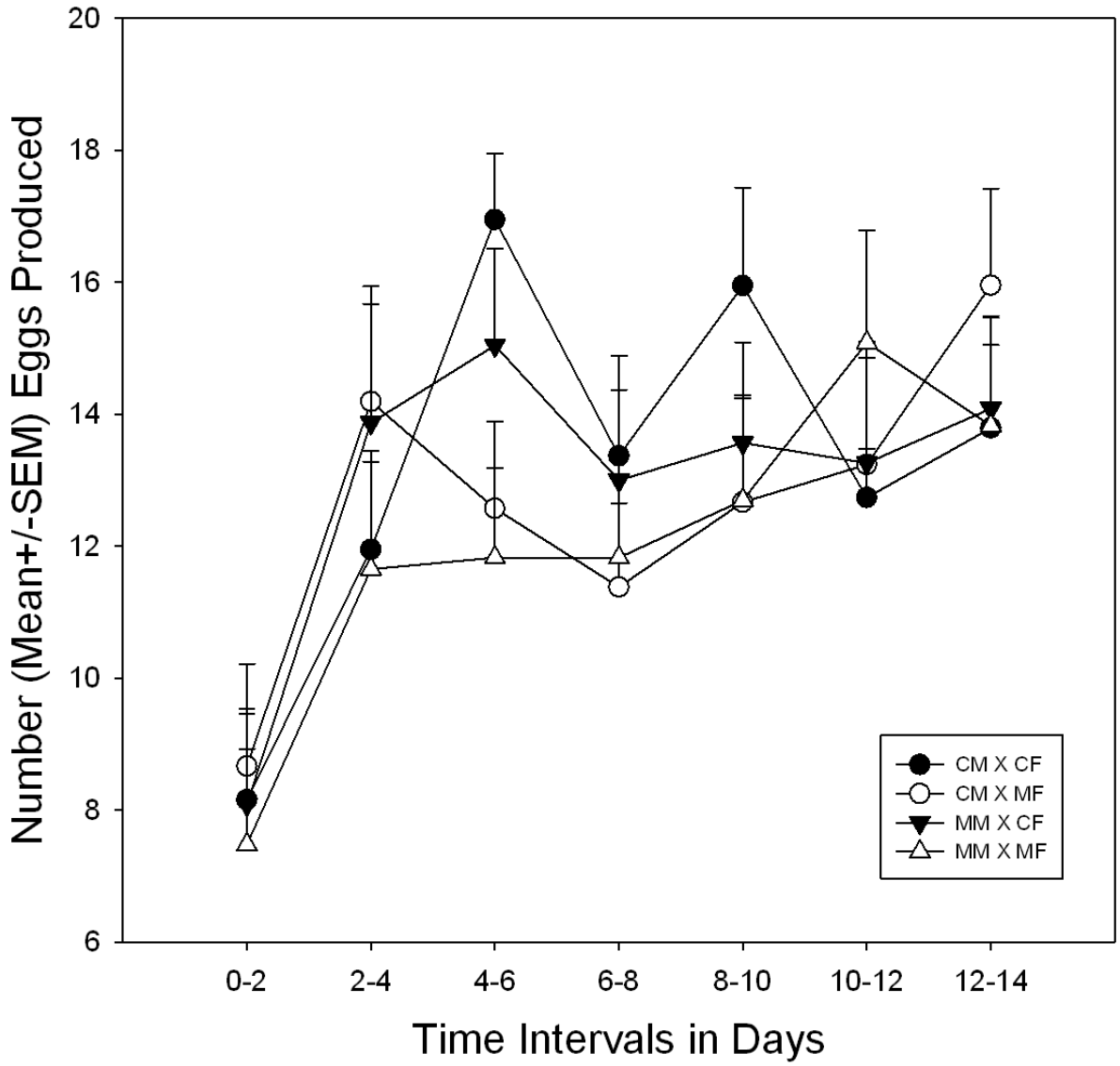

Figure 2.5 The number (mean±SEM) of the number of eggs oviposited by *T. castaneum* adult pairs that were exposed as immatures to flour only (CM-control male, CF-control female, or CM X CF- control) or flour and a methoprene treated adult (MF- methoprene male, MF- methoprene female, or MM X MR- methoprene) over the 14 day observation period; there were no significant differences between treatment means according to least square means test with a Tukey-Kramer multiple comparisons adjustment (P>0.05).

Table 2.1 The number (N) of *Tribolium castaneum* (RFB) and *T. confusum* (CFB) exhibiting deleterious effects (DE), i.e. external deformities and/or mortality during the observation period, after expressing different levels of cannibalization on water treated larvae, pupae, or adults or methoprene treated larvae, pupae, or adults. Level of cannibalization was characterized as Rank 0 when there is no evidence of cannibalization, Rank 1 when there is some evidence of cannibalization such as bites and scratches in the cuticle, Rank 2 when there is an intermediate level (greater than Rank 1 but less than Rank 3) of feeding signified by missing appendages and significant feeding of the head or abdomen, and rank 3 is when the developmental stage is completely consumed.

Species	**Treatment**	**Total N**	**Rank 0**		**Rank 1**		**Rank 2 and 3**	
			N	DE	N	DE	N	DE
T. castaneum	Control	111	84	15%	12	17%	15	20%
	Methoprene	116	71	29%	30	40%	15	60%
T. confusum	Control	110	96	4%	7	14%	7	14%
	Methoprene	119	90	19%	19	68%	10	10%

Chapter 3 - Mechanisms for Horizontal Transfer of Methoprene from Treated to Untreated *Tribolium castaneum* (Herbst)

Abstract

Experiments were performed to determine the mechanisms behind the horizontal transfer of methoprene from treated to untreated *Tribolium castaneum* (Herbst). Individuals exposed to 5 methoprene treated developmental stages had 100% mortality, 0% adult emergence, and a significantly lower survival time compared to the control groups (flour only, no food, or water treated larvae, pupae or adults). This demonstrated horizontal transfer of methoprene between treated and untreated individuals but did not allow for an inference on the mechanism. To evaluate the role of cannibalism on horizontal transfer of methoprene, untreated larvae were allowed to feed on water or methoprene treated pupa and then were ranked and placed in treatment groups according the level of cannibalization. The level of feeding did not effect the normal surviving adults except in the treatment where no food source was provided. Contact between methoprene or water treated adults and an untreated late-stage larva did not reduce the number of normal surviving adults or the survival time indicating that brief exposure time does not allow for horizontal transfer of methoprene. Contact and/or feeding on contaminated substrate was evaluated by treating flour with multiple water or methoprene treated developmental stages. Late-stage larvae in flour treated with methoprene treated developmental stages had significantly higher deformities (84%) and mortality (89%), lower adult emergence (25%), and a lower survival time compared to the controls. These results suggest that the likely mechanism for horizontal

transfer of methoprene is the contact with multiple methoprene treated individuals or contaminated surfaces.

Introduction

Tribolium castaneum (Herbst), the red flour beetle, is a major insect pest found worldwide in food processing facilities. It is difficult for most insecticides to penetrate into the hidden areas inside structural walls and pieces of equipment where *T. castaneum* can live and thrive (Campbell et al., 2004). One common method for treating *T. castaneum* populations in food processing facilities is aerosol applications. Aerosol applications may consist of a single insecticide or combination of insecticides with different modes of action. An example of a commonly used mixed insecticide is methoprene, an insect growth regulator which targets immature insects during development (Henrick, 2007), and synergized pyrethrin, a pyrethrum daisy based insecticide (Soderlund, 1992) which target exposed insects. In the case of food processing facilities it is hypothesized that the adults are more likely to be out in the open and to be exposed during aerosol applications.

Aerosol applications are not expected to have an effect on hidden populations since they only effect individuals which are directly exposed or come in contact with treated surfaces after the aerosol application (Peckman and Arthur, 2006; Arthur, 2008). However, results from mill trapping suggest that a combination of aerosol applications of methoprene and synergized pyrethrin and sanitation measures suppressed *T. castaneum* populations (Campbell and Arbogast, 2004). Horizontal transfer, movement of insecticides from treated to untreated individuals, has been considered as a method of exposing hidden populations to insecticides and has been shown to occur between

treated and untreated *T. castaneum* (Chapter 2). However, the mechanisms for this transfer have not been evaluated. Determining how the transfer of methoprene between treated and untreated individuals could help determine if this is a viable method of treating hidden populations which are not expected to be effected by aerosol applications.

T. castaneum has three biological traits which could facilitate horizontal transfer of methoprene. First, males produce an aggregation pheromone which attracts both sexes (Suzuki, 1980; Suzuki, 1981) and could contribute to the forming of groups which in turn leads to increased contact between individuals. Males directly exposed to aerosol applications or treated surfaces may move into a hidden refugia, secrete aggregation pheromone, attract previously unexposed individuals, and by contact translocate, move, the insecticide to those individuals (Chapter 2).

Second, cannibalization of living and dead individuals is exhibited by larvae and adults (Park et al., 1965) and feeding on insecticide treated individuals could result in both cuticle to cuticle contact and ingestion of insecticide. Individuals exposed to insecticides that cause knock-down or mortality may be vulnerable to feeding by other larvae or adults. This feeding can result in translocation of insecticides from the exposed to previously unexposed individuals (Chapter 2).

Third, adults readily move in and out of flour residues in hidden areas and cross open areas which could facilitate both encounter with treated surfaces and the movement of insecticide into hidden areas. The exploitation of flour residues/patches (Campbell and Hagstrum, 2002; Campbell and Runnion, 2003) allows for the previous two behavioral traits to be more likely to occur and transfer could be facilitate by both

direct contact among individuals or the transfer of the insecticide from the cuticle to the flour where it could be either consumed by feeding individuals or translocated from the flour to the cuticle of individuals moving through the flour.

The objectives of these experiments were to determine the mechanism for the horizontal transfer of methoprene in *T. castaneum*. In a series of experiments the three potential mechanisms were evaluated: effect on development from larvae to adult after contact with a methoprene treated individual, different levels of feeding (cannibalization) on methoprene treated individuals, and exposure to flour previously conditioned with multiple methoprene treated individuals. Successful transfer of methoprene was determined based on the presence of physical deformities, failure to develop to the adult stage, and mortality during the observation period.

Materials and Methods

The following experiments used a *T. castaneum* colony originally obtained from a commercial flour mill in 2004 (Romero et al., 2010). Insect cultures were maintained on a mixture of unbleached wheat flour and brewer's yeast (5% by weight) at 28.34±0.02^{o}C, 46.26±0.08% RH and 14/10 (day/night) photoperiod, with detailed rearing and culture methods described in Chapter 2. Insects used in experiments were collected between three to six weeks after the initial inoculation with adults; the time of collection was based on the developmental stage being collected e.g. five weeks for pupae and late-stage larvae and six weeks for adults. Adult specimens, voucher number 226, were placed in the Kansas State University Museum of Entomological and Prairie Arthropod Research (KSU-MEPAR).

Does contact with and/or feeding on treated individuals result in horizontal transfer of methoprene?

Concrete Petri dish exposure arenas were created as described in Chapter 2. *T. castaneum* adults, pupae, and late-stage larvae were collected from the above mentioned colonies. The developmental life-stages were divided into two groups of thirty; one group was treated with methoprene and the other with distilled water using and artists air brush (Badger Air-Brush Company, Franklin Park, IL, USA) and processed as described in Chapter 2.

Single late-stage *T. castaneum* larvae (focal insects), collected from jars that were five weeks post initial adult inoculation, were exposed to five dead distilled water or methoprene treated developmental stages (larvae, pupae or adults) in polystyrene shell vials (17.7 ml, 7.9 cm long x 1.7 cm wide) (A. Daigger and Co., Inc., Vernon Hills, IL, USA). The treated stages were the only source of food provided to the focal individuals. Two additional control treatments were also setup in the same type of vial. In the first, the single late stage larvae focal individual was provided with 1 g of flour. In the second, the focal individuals were held in the vials without a food source.

There were five vials per treatment per block, day, and three blocks were performed for a total of 15 replicates in each treatment. The focal insect was observed for 30 days mortality, adult emergence, and deformities were recorded. Mortality was defined as death during the observation period, adult emergence was the successful emergence of an adult, and deformities were any external physical malformations i.e. twisted wings, adultoids (transition between pupa and adult), or pieces of previous molts or pupal exuvia attached to the cuticle.

The level of cannibalization that occurred was quantified using an index that ranged from zero to three. A rank of 0 indicated no cannibalization signifying no attempts to feed, rank 1 had scratches on the cuticle and/or one or two bites in cuticle signifying an attempt to feed, rank 2 was any signs of feeding between ranks 1 and rank 3 such as complete consumption of the head capsule or significant feeding damage to the abdomen, and rank 3 was complete consumption of the developmental stage. Each developmental stage was assigned a rank and the average of the five individuals was used for analysis. The average cannibalization rank of the five larvae, pupae or adults added to the vials was analyzed for each treatment group using the generalized liner model procedure (SAS software v. 9.2, SAS Institute, Cary, NC, USA) to determine if the amount of cannibalization was effected by the treatment and developmental stage provided.

A Kaplan-Meir log-rank survival analysis (SigmaPlot, v. 11.0 software, Systat Software Inc., Chicago, IL, USA) was used to evaluate the survival of focal insects throughout the experimental period. Holm-Sidak method was used to perform multiple pairwise-comparisons when significant differences were identified.

GLIMMIX analysis (SAS software v. 9.2, SAS Insititue, Cary, NC, USA) was used to analyze the number of focal insect which after exposure and feeding were normal surviving adults; this measure accounts for of all the possible deleterious effects: external deformities, inability to successfully emerge as adults, and mortality during the observational period. GLIMMIX is a generalized linear mixed model that can be used to analyze binary data. Each analysis required a different random statement based on where the random effects occurred; the experimental block, replicate, and/or interaction

between the block and the replicate were included as a random variable in the model. The control (individuals exposed to flour only, no food, and water treated individuals) and the methoprene (individuals exposed to methoprene treated individuals) treatments were analyzed separately to determine significant differences and then were compared to each other to determine significant differences between the control and methoprene treatments. Tukey –Kramer grouping for least square means was included in the analysis to provide information on the significance of the means separation tests.

Does level of cannibalization on dead, methoprene treated pupae effect development of previously unexposed individuals?

T. castaneum pupae were collected, treated with distilled water or methoprene, and processed as described above. One late-stage larva, the focal insect, was exposed to dead distilled water or methoprene treated pupa, in a Petri dish (90 x 15 mm) for 48 hours. After the 48 hours, the level of cannibalization by the larva was assessed and ranked according to the previously described cannibalization scale. Larvae which pupated during this period were eliminated. Focal larvae were assigned to groups based on the cannibalization rank and transferred to new Petri dishes with 1 g of flour and 5% Brewer's yeast (by weight). In addition to the water treated control, two additional controls were included with each block, day: individuals provided with flour only and individuals provided with no food. There were two blocks performed, with 21-25 individuals per treatment combination in each block, which resulted in 43-46 individuals in each treatment group. The focal insects were examined 3-5 times a week for 30 days for deformities, adult emergence, and/or mortality. The time to death, survival analysis, and normal surviving adults were analyzed as described above.

Can contact with treated individuals result in horizontal transfer of methoprene to previously unexposed individuals?

Adult *T. castaneum* were collected and treated as described above. Late-stage larvae (focal insects) were placed individually on the bottom of a plastic Petri dish (90 x 15 mm) containing a tent refuge area made from a folded piece of 20 mm X 10 mm filter paper (Whatman Grade 1, GE Healthcare Co., Little Chalfont, Buckinghamshire, UK) and one living methoprene or water treated adult. After twenty-four hours, the focal larva was removed from the Petri dish and placed in a new Petri dish with 1 g of flour and a new paper tent. The larva was then observed for thirty days for presence of deformities, adult emergence, and/or mortality in order to determine the normal, surviving adults at the end of the experiment. There were four blocks, day, with 6-10 replicates per treatment combination in each block; resulting in a total of 25-31 replicates per treatment combination. The resulting data was analyzed as described above.

Can methoprene be transferred from treated individuals to flour substrate and then to another individual?

T. castaneum larvae, pupae, and adults were collected, treated with distilled water or methoprene, and processed as described above. Groups of 5, 15, or 30 dead individuals were placed in 17.7 ml polystyrene shell vials with 1 g of flour. The vials were shaken for 30 minutes on a bench-top orbital shaker (OR25, A. Daigger and Co., Vernon Hills, IL, USA) set at 200 rpm. The shaker movement agitated the flour in the vials and this movement was used to simulate beetles moving through the flour. The treated individuals were then removed by sieving with a size 30 mesh sieve and 1 late-stage larva (focal individual) was added to the flour. The focal individual was held in the

flour for 30 days and observed daily for presence of physical deformities, adult emergence, and/or mortality; this information was used and analyzed as described above. There were three blocks, day, with 2-5 replicates for each treatment combination in each block; 9-12 total replicates for each treatment combination.

Results

Does contact with and/or feeding on treated individuals result in horizontal transfer of methoprene?

The survival analysis for the *T. castaneum* late-stage larvae exposed to flour only (untreated control), no food, or five water treated developmental stages (larvae, pupae, or adults) showed significant differences between the control treatments ($Z=20.164$, d.f.=4, $P<0.001$). The Holm-Sidak pair-wise comparison test showed that the survival of focal insects exposed to flour was significantly different from the no food treatment, but none of the water treated stages were significantly different from any of the other control treatments. The three water treated development groups were grouped together but the untreated control and no food treatments were left independent for further analysis. Late-stage larvae exposed to methoprene treated developmental stages (larvae, pupae, or adults) were not significantly different ($Z=3.050$, d.f.=2, $P=0.218$) from each other and were combined for further analysis. Analysis of the newly grouped data indicated significantly differences among treatments ($Z=49.788$, d.f.=3, $P<0.001$) (Fig. 3.1). Exposure to methoprene treated individuals resulted in more rapid mortality compared to exposure to the combined controls.

Individuals that were secondarily exposed to methoprene had 100% mortality and no adult emergence, regardless of the developmental stage provided. Because all

the individuals responded it was not possible to analyze the normal surviving adult data using GLIMMIX due to the limitations of the binary GLIMMIX model. *T. castaneum* exposed to water-treated adults or to no food also had high mortality (67% and 79%, respectively) and low adult emergence (67% and 65%, respectively). Individuals provided with flour had low mortality (20%) and high adult emergence (93%). This suggests that some of the mortality in the methoprene treatments was due to starvation which was a result of limited feeding on the provided insects. Additionally, the average amount of cannibalization was significantly different among the six treatments ($F=8.33$; d.f.=5, 84; $P<0.001$) (Table 3.1). Observed cannibalization was lowest on the adult stages, whether water or methoprene treated, likely due to hardness of adult cuticle.

The percentage of individuals exhibiting deformities was analyzed using GLIMMIX. The percentage of deformities not different among the different controls ($F=1.61$; d.f.=2, 58; $P=0.2083$) or among the methoprene treatments ($F=0.64$; d.f.=2, 36; $P=0.5339$) so they were combined for further analysis. The combined methoprene treatments (68.25%, n=44 with deformities) was significantly greater ($F= 27.54$; d.f.=1, 104; $P<0.0001$) than the combined control (9.62%, n=74 with deformities) treatments according to GLIMMIX and Tukey-Kramer multiple comparison test.

Does the level of cannibalization on dead, methoprene treated pupae effect the development of previously unexposed individuals?

T. castaneum focal insects that were exposed to flour or were allowed to feed on a water or methoprene treated pupa in order to determine cannibalization rank had similar percentage of individuals with deformities, adult emergence and mortality. The lack of significant differences in the normal, surviving adults and the survival time

indicate that the level of cannibalization did not result in deleterious effects. The only treatment that had a significant effect was the no food treatment. Individuals that were exposed to no food had a higher percentage of mortality and deformities and a lower percentage of adult emergences compared to the other treatments. The survival of the focal insects after exposure to no food was significantly lower compared to all other treatment groups ($Z=273.459$, d.f.=9, $P<0.001$) (Fig. 3.2). The number of *T. castaneum* late-stage larvae that were normal surviving adults at the end of the observation period after exposure to no food was also significantly different compared to all other treatments ($F=8.11$; d.f=9, 411; $P<0.0001$) (Fig. 3.3).

Can contact with treated individuals result in horizontal transfer of methoprene to previously unexposed individuals?

The survival analysis for focal insects that were exposed to water or methoprene treated adults or unexposed to treated adults (untreated control) showed no significant differences ($Z=0.187$, d.f.=2, $P=0.911$) (Fig. 3.4) in the rates of mortality between the three treatment groups. The percentage of normal surviving adults after exposure to flour only (80%) or to a water (94%) or methoprene (69%) treated adult was also not significantly different ($F=2.13$; d.f=2, 59; $P=0.1275$). This result suggest that brief exposure times are not enough for horizontal transfer of methoprene to result in detectable deleterious effects such as mortality and/or external deformities.

Can methoprene be transferred from treated individuals to flour substrate and then to another individual?

The survival time analysis was simplified as described above. *T. castaneum* focal insects exposed to flour only or flour that was conditioned with 5, 15, or 30 water treated

larvae, pupae or adults (Z=0.451, d.f.=2, P=0.798; Z=1.947, d.f.=2; P=0.378; Z=0.185, d.f.=2, P=0.912, respectively) or methoprene treated larvae, pupae, or adults (Z=0.964, d.f.=2, P=0.618; Z=0.667, d.f.=2, P=0.735; Z=0.905, d.f.=2, P=0.086, respectively) were not significantly different so the control and methoprene treatments were combined for further analysis. The survival time for focal individuals exposed to flour conditioned with 5, 15, or 30 methoprene developmental stages was significantly lower than focal insects in flour only or flour that was conditioned with 5, 15, or 30 water treated developmental stages (Z=138.043, d.f.=6, P<0.001) (Fig. 3.5).

None of the *T. castaneum* late-stage larvae exposed to flour conditioned with 15 or 30 methoprene treated larvae, pupae, or adults had normal surviving adults. Therefore, this data could not be analyzed due to the limitations of GLIMMIX. *T. castaneum* late-stage larvae exposed to 5 methoprene treated adults, also, had 0% normal surviving adults but those that were exposed to 5 methoprene treated larvae or pupae both had 10% normal surviving adults. Individuals that were exposed to the control conditions had a greater percentage of normal surviving adults: flour only (70%), 5 water treated developmental stages (64-72%), 15 water treated developmental stages (45-73%), and 30 water treated developmental stages (64-73%).

The percentage deformities observed was analyzed with GLIMMIX. There were no significant differences in deformities among focal individuals exposed to flour treated with multiple numbers of water treated developmental stages (F=0.47; d.f.=2, 75; P=0.6275) so they were combined with the flour only treatment, which could not be analyzed due to no observed deformities, for further analysis. Multiple treatment groups where focal insects were exposed to flour treated with methoprene treated

developmental stages (late-stage larvae, pupae, or adults) had 100% deformities. Therefore, the developmental stages at each level of flour conditioning (5, 15, or 30 developmental stages) were combined and compared to the combined (flour only or flour treated with 5, 15, or 30 water treated developmental stages). The percentage of focal insects with physical deformities after exposure to flour conditioned with 5 (84%), 15 (87%), or 30 (80%) methoprene treated developmental stages was significantly greater than for focal insects exposed to flour only or flour conditioned with 5, 15, or 30 water treated developmental stages (12%, combined controls) ($F=21.64$; d.f.=3, 179; $P<0.0001$).

Discussion

Previous studies exploring horizontal transfer of insecticides between treated and untreated insects generally focused on insecticide efficacy (Aubuchan et al., 2006) and/or the insecticide delivery method (LePatourrl, 1999; Buczkowski et al., 2001; Buczkowski and Schal, 2001). Although insect behaviors such as cannibalization (Le Patoural, 1999; Buczkowski et al., 2001; Buczkowski and Schal, 2001, necrophagy, and/or copraphagy (Buczkowski et al., 2001; Buczkowski and Schal, 2001), and contact (LePatourel, 1998) have been attributed to causing horizontal transfer, the specific mechanism of transfer was not the focus of these experiments. The current experiments determined that horizontal transfer of methoprene was facilitated by lengthy contact times and multiple exposures.

Contact appears to be an important mechanism of transfer but the time of exposure appeared to have a major effect on efficacy. Only in experiments with long exposure times or exposure during a critical point in development had strong effects

detectable effects of horizontal transfer. In the contact only experiment no detrimental effects of exposure to methoprene treated adults was observed. However, physical contact between the two individuals may have been limited by the length of exposure time, arena size, and their respective activity patterns. Tarsal contact in a larger arena potentially aided in horizontal transfer of cypermethrin in Oriental cockroaches (LePatourel, 1998). This suggests that not only the size of the arena but what is treated with insecticides could aid in the horizontal transfer of insecticides.

In the other experiment which included both contact and cannibalization, there were strong treatment effects that appeared independent of amount of feeding. This suggests that the failure to detect horizontal transfer in the contact only experiment may be because of the brief exposure time not allowing sufficient transfer to generate detectable effects (i.e., deformities and mortality). The insects were not observed during the 24 hour exposure time and it is possible that there was little or no contact or interaction between the methoprene treated adults and late-stage larvae. At the time of transfer, adults were frequently observed walking in circles at the Petri-dish boundary, but the larvae were frequently on or near the paper tent refuge (personal observation). Future experiments focused on length of exposure and efficacy would help in the evaluation of these results.

Cannibalism did occur and the level of feeding was highly variable, however, most of the effects of these experiments appear to be due to starvation and not the transfer of methoprene. These results support findings in Chapter 2 and suggest that cannibalization is not a mechanism for the horizontal transfer of methoprene between treated and untreated *T. castaneum*. However, in some cockroach species

cannibalization has been determined to be an important factor in horizontal transfer of insecticides (Le Patourel, 1999; Buczkowski et al., 2001; Buczkowski and Schal, 2001). Le Patourel (1999), Buczkowski et al. (2001), and Buckzkowsi and Schal (2001) were able to quantify the amount of insecticide the cockroaches consumed when feeding on poisoned cadavers and then determine the effect on mortality. In our studies we have not been able to directly quantify the amount of methoprene on treated individuals or how much is transferred. This is partly due to the low amounts of methoprene present which makes detection difficult. However, bioassay data can be used to detect low levels of methoprene and in some of the experimental conditions tested bioassay results indicate transfer (Chapter 2). Further research is needed to quantify the amount needed to result in deleterious effects.

The strongest effects of methoprene appeared to be through the transfer of methoprene from insect cuticle to the flour and then translocation through either contact or feeding to untreated individuals. This experiment also involved a long period of exposure to the treated flour, which supports the idea that length of exposure is important for efficacy either due to cumulative effects or presence during critical physiological points in development. Additionally, the length of exposure to insecticides has been shown to affect survival of lesser grain borer, *Rhyzopertha dominica* (F.) exposed to diatomaceious earth (Vardeman et al., 2006) and larval developmental time and egg and larval mortality of the Indianmeal moth, *Plodia interpunctella* (Hübner) exposed to hydroprene (Mohandass et al., 2006ab)

The flour was exposed to different numbers of methoprene treated individuals for 30 minutes. During the exposure time, the flour was agitated vigorously; this action

could have increased the amount of methoprene that was removed from the insect cuticle to the flour. The focal larva was exposed to the treated flour for 30 days. A small proportion of individuals were able to develop into adults in the flour that was treated with 5 methoprene treated developmental stages. The effects of secondary exposure to methoprene were much higher than previously reported (Chapter 2) but the insects used in the current study were from a more recently collected colony which may have an effect of the efficacy of methoprene.

The role of flour in absorbing methoprene and transferring it to insects is an interesting and potentially important observation. This mechanism may be important not only for horizontal transfer, but also because movement of flour or other food material in a food facility may transport methoprene as well. Flour or other food materials could be exposed to methoprene during the aerosol application process or given the propensity of flour to absorb methoprene, by settling on treated surfaces post aerosol application. Over time treated flour can be moved around by sanitation activities such as sweeping, vacuuming, or the use of compressed air. These activities could introduce treated flour into hidden areas such as cracks and crevices where pest populations are located. These results raise another possible mechanism for movement of methoprene in hidden areas that might contribute to the populations suppression effects previously reported (Campbell and Arbogast, 2004).

The length of exposure time to small amounts of methoprene seems to be driving the deleterious effects seen in these experiments and in previous work (Chapter 2). However, these studies focus on the susceptibility of individual *T. castaneum* to the secondary exposure of methoprene. This susceptibility may or may not result in an

effect on *T. castaneum* populations. Additionally, only single treatments were performed but in real-world flour mills aerosol applications that include methoprene are performed routinely and thus the chances of transfer are potentially increased. Future work on the horizontal transfer of methoprene from treated to untreated *T. castaneum* should evaluate these individual effects at the population level and consider more realistic flour mill management practices i.e. sanitation and routine aerosol applications of synergized pyrethrin formulated with methoprene.

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Figures and Tables

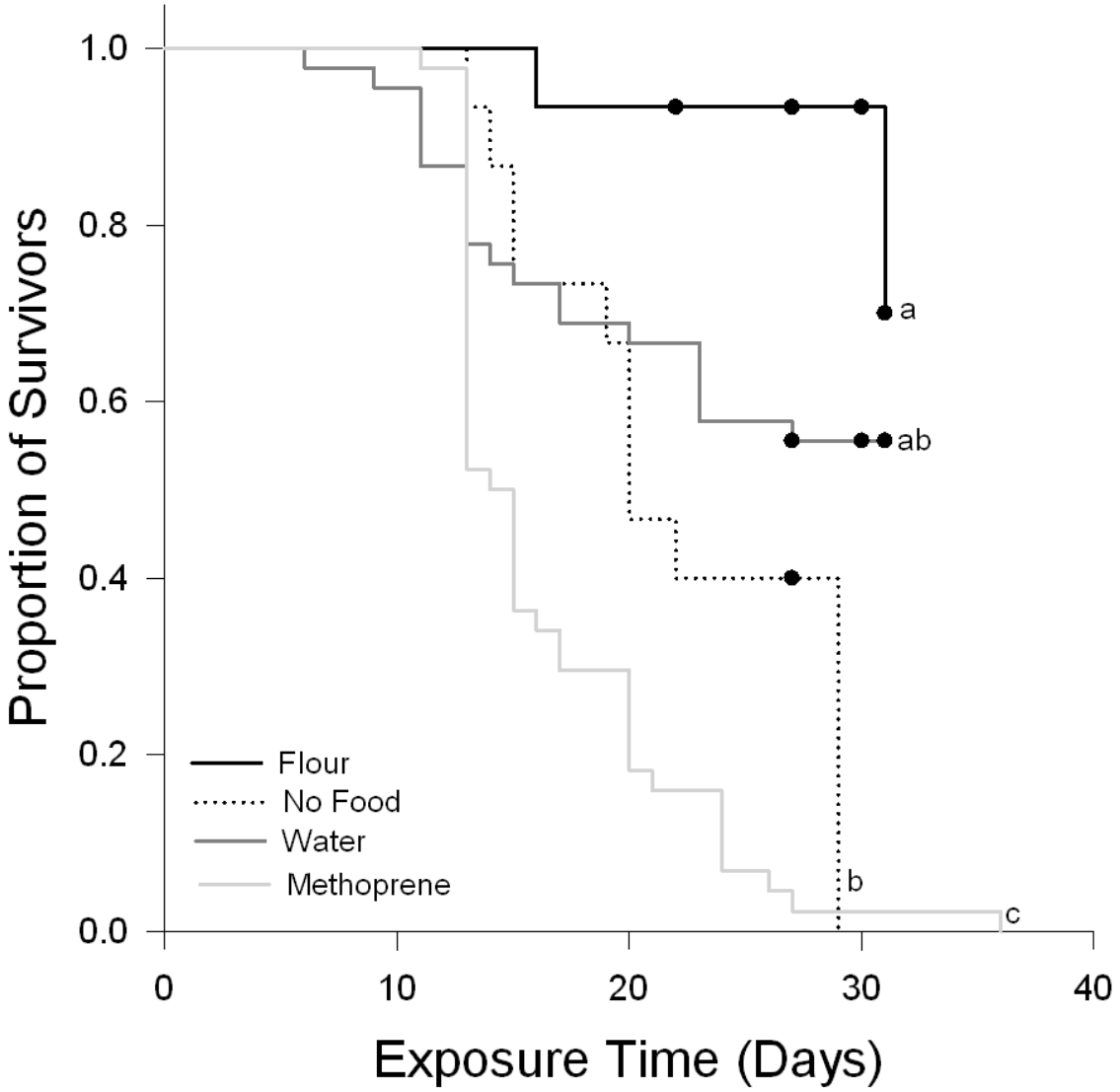

Figure 3.1 The survival curves for late-stage *T. castaneum* larvae after exposure to flour only (control), no food, 5 water treated developmental stages (Water), or 5 methoprene treated developmental stages (Methoprene); the black circles on the survival curve are where the experiment was terminated and each step down from the line indicates a proportion of the replicates which died on that day. Same lowercase letters next to the survival line indicate no significant pair-wise comparisons between treatments according to Holm-Sidak.

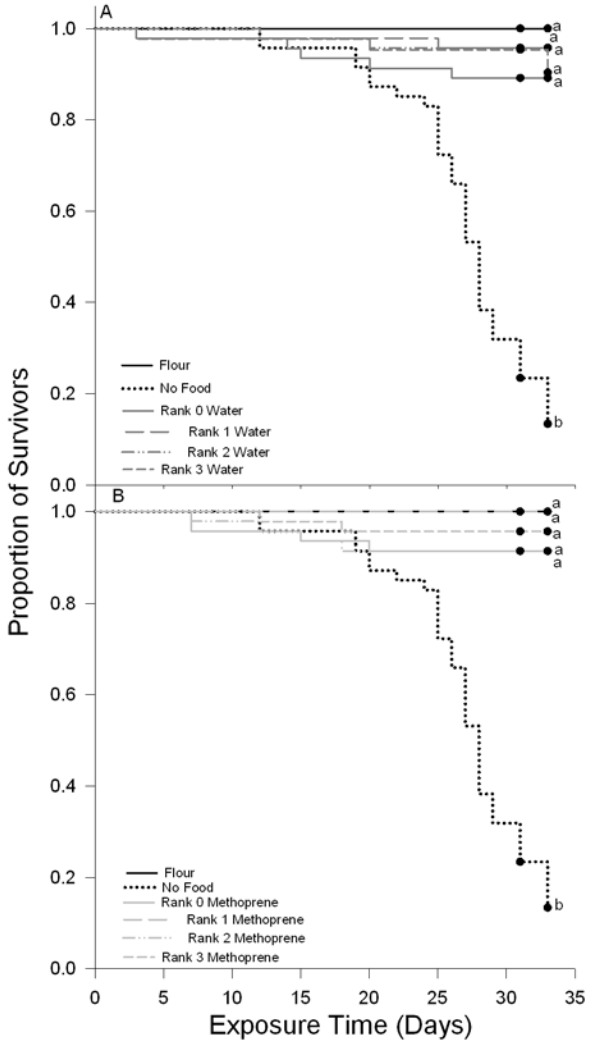

Figure 3.2 The survival analysis for *T. castaneum* late-stage larvae after exposure to flour, no food, and a 48 hour period where individuals were allowed to feed and contact water (A) or methoprene (B) treated pupa and then were ranked by the cannibalization rank where 0 = no feeding, 1= one or two bites or scratches in the cuticle, 2= intermediate feeding between ranks 1 and 3 i.e. missing appendages and/or significant feeding on abdomen, and 3 = complete consumption of the individual. The black circles on the survival curve are where the experiment was terminated and each step down from the line indicates a proportion of the replicates which died on that day. Same lowercase letters next to the end of the survival line indicate no significant pair-wise comparisons according to Holm-Sidak.

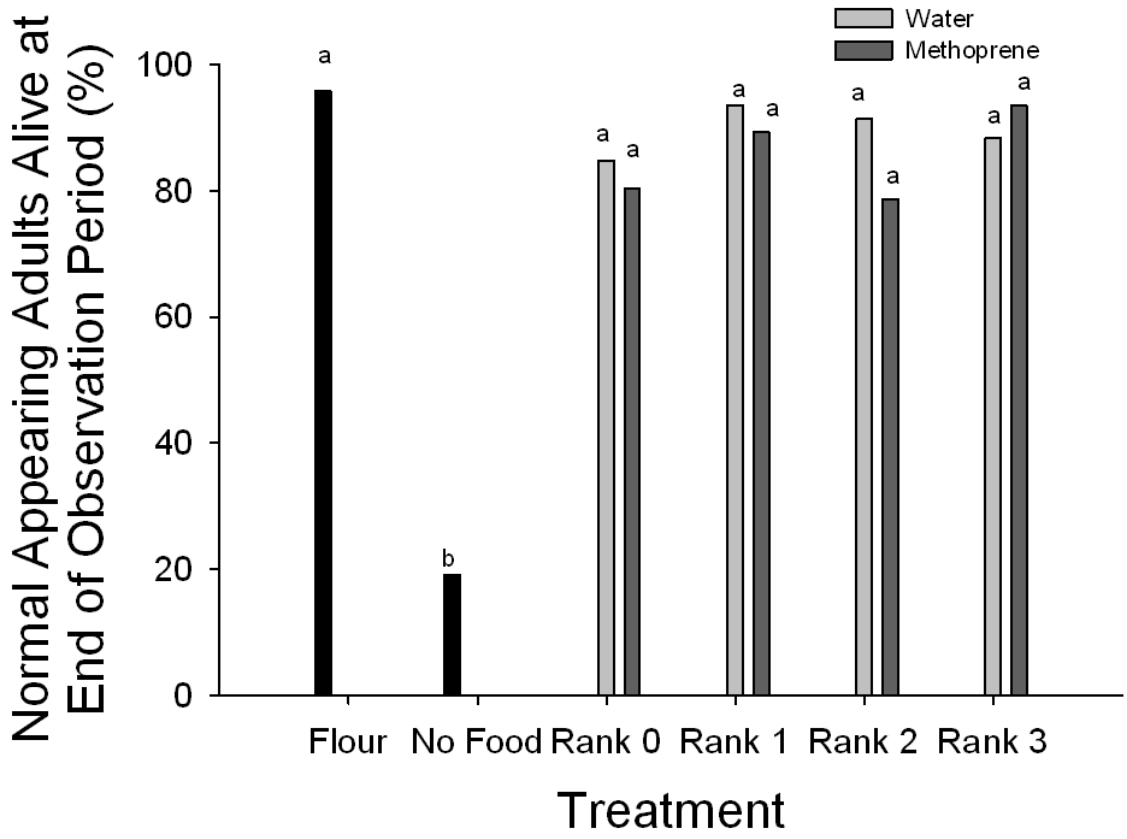

Figure 3.3 The percentage of normal, adults surviving after exposure to flour only, no food, and a 48 hour period where individuals were allowed to feed on and contact a water or methoprene treated pupa and then were ranked by an established cannibalization rank where 0 = no feeding, 1= one or two bites or scratches in the cuticle, 2= intermediate feeding between ranks 1 and 3 i.e. missing appendages and/or significant feeding on abdomen, and 3 = complete consumption of the individual. Different lower-case letters above the bars indicate significant differences across cannibalization ranks according to Tukey-Kramer least means multiple comparison test.

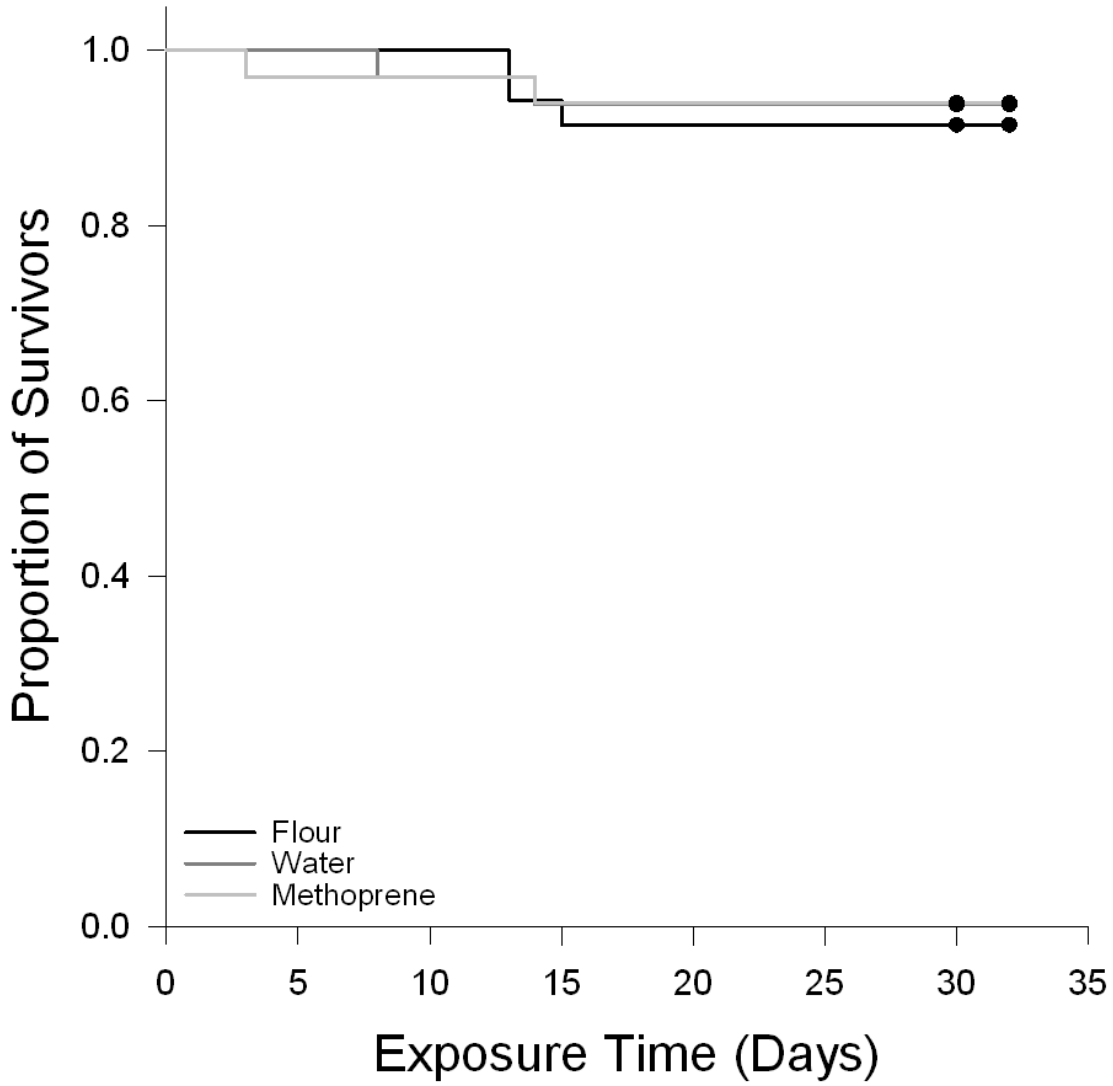

Figure 3.4 The survival analysis for *T. castaneum* late-stage larvae after a 24 hour exposure period where contact with water or a methoprene adult could occur. The black circles on the survival curve are where the experiment was terminated and each step down from the line indicates a proportion of the replicates which died on that day. There were no significant pair-wise comparisons between the treatment survival lines according to Holm-Sidak.

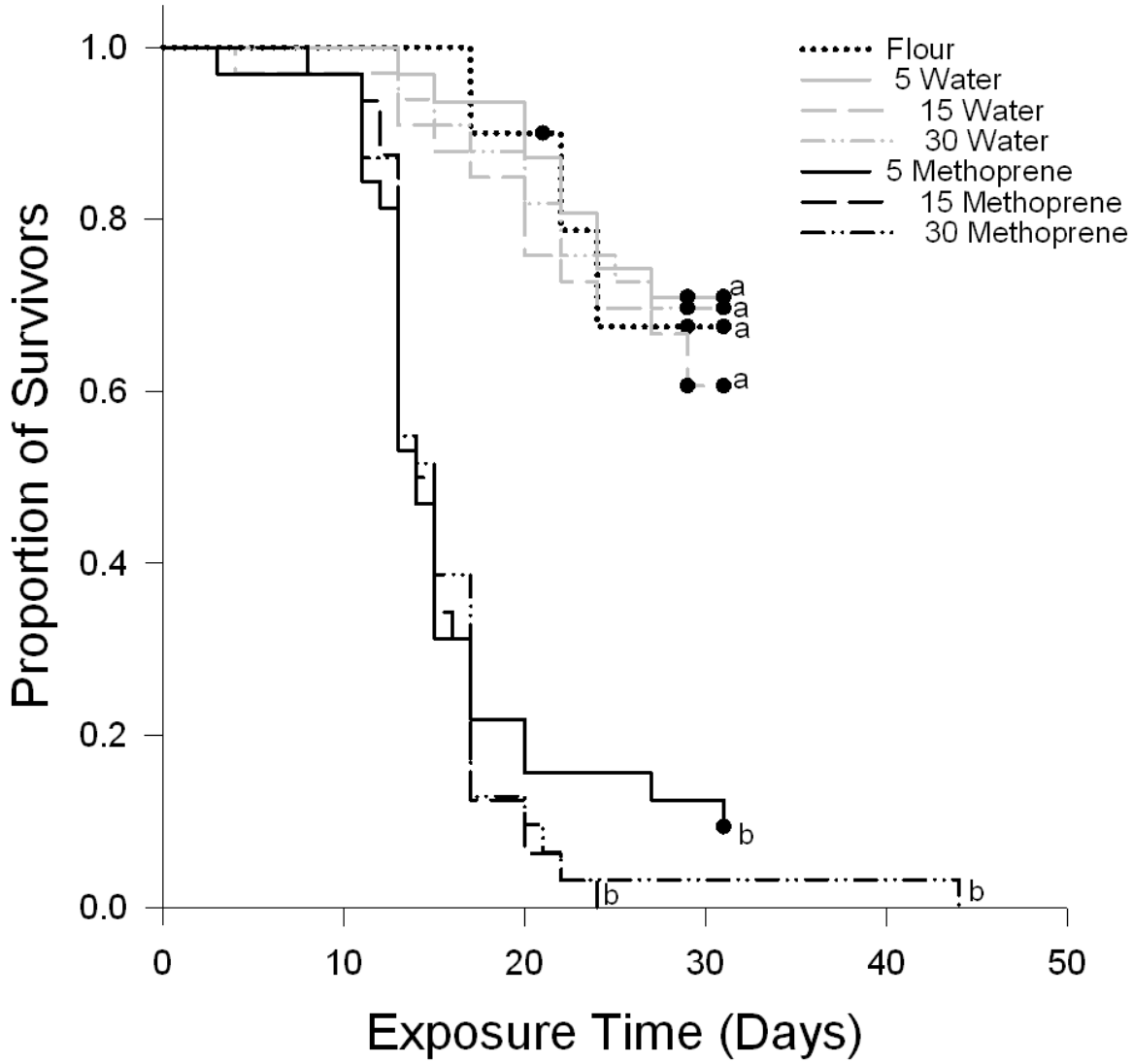

Figure 3.5 The survival analysis for *T. castaneum* late-stage larvae after exposure to flour only or flour that was conditioned with 5, 15, or 30 developmental stages (larvae, pupae, or adults) which were treated with water or methoprene. The black circles on the survival curve are where the experiment was terminated and each step down from the line indicates a proportion of the replicates which died on that day. Different lower case letters next to the end of the survival line indicate significant pair-wise comparisons according to Holm-Sidak.

Table 3.1 The number of individuals which exhibited the level of cannibalization ranking (based on the average of 5 individuals) and the percentage of individuals that exhibited deleterious effects (DE) (i.e. mortality and/or deformities) of *T. castaneum* late-stage larvae after cannibalizing and/or contact with 5 water or methoprene treated developmental stages (n= 15).

Treatment	Rank 0		Rank 1		Rank 2and3	
	N	DE	N	DE	N	DE
Water Larvae	2	50%	9	56%	4	0%
Water Pupae	1	0%	7	14%	7	14%
Water Adults	13	69%	2	100%	0	0%
Methoprene Larvae	6	100%	3	100%	6	100%
Methoprene Pupae	2	100%	3	100%	7	100%
Methoprene Adults	10	100%	5	100%	0	0%

Chapter 4 - Efficacy of Aerosol Applications of Methoprene and Synergized Pyrethrin against *Tribolium castaneum* (Herbst) Adults and Eggs

Abstract

Experiments were performed to determine the efficacy of the insecticides methoprene and synergized pyrethrin, alone or in combination, and that of the carrier, Isopar M which is commonly used in formulations. *T. castaneum* adult survival and number of living F1 progeny produced was not significantly affected by exposure to any of the treatments: untreated control or an aerosol application of Isopar M; methoprene and Isopar M; synergized pyrethrin and Isopar M; or both insecticides and Isopar M. This suggests a low efficacy for single aerosol applications directly on adults. Egg hatch was suppressed by methoprene, synergized pyrethrin, and methoprene and synergized pyrethrin (all formulated with Isopar M) compared to the controls but synergized pyrethrin was the most effective ($P<0.001$). Flour residues on the eggs effected both the insect development and insecticide efficacy. Low amounts of flour tested were insufficient for individuals to develop beyond the early larval stages even in the controls. As the depth of flour increased, the number of individuals that could develop to the adult stage increased but this was lower in the insecticide treatments than in the control or carrier treatments. These results indicate a high efficacy of the individual insecticides and the combination of methoprene, synergized pyrethrin, and Isopar M on *T. castaneum* egg hatch and development to the adult stage but there are still questions regarding the efficacy on adult *T. castaneum*.

Introduction

Tribolium castaneum (Herbst) is a cosmopolitan stored product insect pest, which can be found in warehouses, pet food stores, and grain processing facilities such as rice and flour mills. They are considered a major insect pest species since they are frequently the least susceptible of stored-product pest species to insecticides (Arthur, 2008). Integrated pest management (IPM) programs that incorporate tactics such as aerosol insecticide applications and improved sanitation measures are widely used in the food industry, but information on the efficacy of these tactics is still limited.

Aerosol applications involve dispersal of an insecticide as small droplets under pressure using either a permanently installed system or a portable applicator (Peckman and Arthur, 2006). Insecticides applied as an aerosol can provide good coverage of exposed surfaces, but they cannot penetrate into commodities or obstructed areas like fumigants (Peckman and Arthur, 2006; Arthur, 2008). A commonly used aerosol insecticide application mixture, synergized pyrethrin and methoprene, has been found to effectively reduce *T. castaneum* populations when used in conjunction with fumigation and sanitation tactics (Campbell et al., 2010ab). Horizontal transfer of methoprene between treated and untreated *T. castaneum* and migration of the insecticide into flour has been demonstrated to increase the potential efficacy of methoprene (Chapters 2 and 3), but this effect has not been evaluated under real-world scenarios nor for potential population effects.

The combination of methoprene and synergized pyrethrin is hypothesized to be effective since they have different modes of action. Methoprene affects immature development by delaying developmental development, causing deformities (Henrick, 2007), reducing survival to the adult stage (Loschiavo, 1974; Williams and Amos, 1974;

Arthur, 2001; Arthur and Hoernemann, 2004), and causing reduced fertility in the surviving adults (Loschiavo, 1974; Oberlander et al., 1997; Chanbang et al., 2007). Synergized pyrethrin, on the other hand, provides immediate knock-down on adults and mortality against all developmental stages (Jensen et al., 2010). The efficacy of synergized pyrethrins has been evaluated for *T. castaneum* adults (Arthur, 2008) and eggs (Toews et al., 2010). Aerosol applications of methoprene on *T. castaneum* late-stage larvae and pupae resulted in 0% adult emergence (Arthur, 2008). These studies provide evidence for the efficacy of the individual components of the combination methoprene and synergized pyrethrin on their target developmental stages but do not evaluate the combination. Additionally, the carrier, Isopar M, is a solvent produced by distilling petroleum which has been found to effect adult emergence of *Plodia interpunctella* (Hübner) eggs (Jensen et al., 2010) and therefore it should be evaluated to determine its effect on *T. castaneum* developmental stages.

Different levels of flour, simulating different levels of sanitation, have been shown to affect the mortality and efficacy of heat treatments (Brijwani et al., 2012) and aerosol applications of synergized pyrethrin (Toews et al., 2010). Additionally, routine aerosol applications of methoprene and synergized pyrethrin used in conjunction with sanitation measures have been shown to reduce *T. castaneum* populations inside flour mills (Campbell and Arbogast, 2004). These studies suggest the potential of sanitation for effecting insect development and insecticide efficacy. However, the effect of sanitation on methoprene and synergized pyrethrin on eggs alone has not been investigated and knowing the potential effects of using these two strategies as part of IPM programs can help insect pest managers make better management decisions.

In this series of experiments the efficacy of the carrier, Isopar M; methoprene and Isopar M; synergized pyrethrin and Isopar M; and the combination of methoprene, synergized pyrethrin, and Isopar M was evaluated for *T. castaneum* adults and the potential effects on resulting F1 progeny. The effect of exposure to these insecticide combinations on *T. castaneum* egg hatch and development after hatch was also evaluated. Because eggs are typically found associated with flour, the effect of different levels of flour accumulation on development and insecticide efficacy was also evaluated.

Materials and Methods

A strain of *T. castaneum* originally collected in 2004 at a local commercial flour mill (Romero et al., 2010) was used in the below experiments. Insect cultures were maintained at 28.0±0.0^{o}C, 46±0.1% RH, and 14/10 (day/night) photoperiod in an incubator (I-36 Series Incubator, Percival Scientific Inc., Perry, Iowa, USA). A detailed description of the routine culture procedures was described in Chapter 2.The Kansas State University Museum of Entomological and Prairie Arthropod Research (KSU-MEPAR) has insect specimens from this research under voucher number 226.

Do different components of methoprene and synergized pyrethrin aerosol application effect adult survival and progeny production?

Groups of males and females were created by sexing adults of mixed-ages using the presence, male, or absence, female, of the setiferous patch as a character (Hinton, 1942). Approximately 20 males and 20 females were placed on separate 90 x 15 mm (62 cm^{2}) Petri dishes with a concrete bottom prepared as described in Arthur (2008). Dishes with insects were then added to a larger plastic box so that simulated aerosol

insecticide application could be applied. The boxes had a layer of concrete on the bottom and after addition of concrete had interior dimensions of 56.5±0.2 cm length by 34.6±0.3 cm width by 15.5±0.1 cm height. A separate box was used for each insecticide treatment.

Adult *T. castaneum* were exposed to simulated aerosol applications of Isopar M (Entech Systems, Kenner, LA, USA); methoprene (Diacon® II, Central Sciences International, West Schaumberg, IL, USA) formulated in Isopar M; synergized pyrethrin and Isopar M (Entech Fog-10, Entech Systems, Kenner, LA, USA); the combination of methoprene, synergized pyrethrin, and Isopar M; or received no aerosol applications (untreated control); water applications were not used since previous work resulted in no differences between the untreated controls and those that were sprayed with water (Chapters 2 and 3). The amount applied to each box was calculated to represent the amount that would settle on a surface during an aerosol insecticide application in a food facility (118.0 m length X 190.2 m width X 30.5 m height were the dimensions of the representative facility).

The dimensions of the representative facility resulted in the following amounts applied to the volume of the representative facility for each treatment: synergized pyrethrin formulated in Isopar M was a total volume of 714 ml but the ratio of synergized pyrethrin to Isopar M is ~10% so 71.4 ml of synergized pyrethrin in 706.9 ml of Isopar M and 0.48 ml of methoprene was used. Since not all of the aerosol will settle to the floor during a commercial aerosol application, we estimated that 25% would not settle on the floor but would be lost during aerosol application. These calculations resulted in 0.5 ml applied to each box; boxes which were treated with Isopar M received only 0.5 ml of

Isopar M which has no active ingredients (a.i.), boxes that were treated with methoprene formulated in Isopar M received 0.05 ml methoprene which translated to 0.0045 ml a.i./cm^2 and 0.45 ml of Isopar M, boxes which received synergized pyrethrin were treated with 0.05 ml synergized pyrethrin which translated to 0.016 ml/cm^2 a.i. and 0.45 ml of Isopar M, and the boxes which received the combination of the two insecticides received 0.05 ml synergized pyrethrin in 0.45 ml Isopar M and since the methoprene is a tiny amount it was added to the synergized pyrethrin in Isopar M formulation and well mixed. The insecticide treatments were applied evenly to the concrete surface of the box, including the dishes containing the insects, using an artist's air-brush (Badger Air-Brush Company, Franklin Park, IL, USA). The dishes were left in the containers for one hour after spraying and then the dishes were removed and covered.

One male and one female from the same treatment were placed in 90 x 15 mm (62 cm^2) Petri dishes with 5.02 ± 0.01 g of flour. Ten dishes with male/female pairs were prepared for each treatment. Knockdown, a condition in which insects are laying on their back with legs up and they respond slowly to stimulation such as prodding with forceps, was determined immediately after the ventilation period and again 24 hours after the aerosol application; mortality was assessed daily for fifteen days. Fifteen days after the aerosol application the adults were removed from the flour by sieving with a size 30 mesh sieve and a final mortality determination was made at that time. The flour was held for an additional 30 days to enable any progeny to develop; the flour was sieved using a size 60 sieve (0.25 mm mesh sieve) (W.S. Tyler Co., Mentor, OH, USA) to remove eggs and then sieved a second time using a size 30-mesh sieve to remove

larvae and pupae. The total number of living, dead, and deformed (i.e., external abnormalities such as twisted wings and previous molts and pupal exuvia attached to cuticle), progeny (eggs, larvae, pupae, and adults) was counted at the end of the experiment. This procedure was repeated three different times, blocks, using different sets of treated individuals, resulting in 30 replicates per treatment.

The treated adult mortality data was analyzed using the GLIMMIX procedure (SAS software v. 9.2, SAS Institute, Cary, NC, USA), a generalized linear model using binary (alive/dead) data. The progeny data, total number of living individuals regardless of developmental stage, was analyzed using a generalized linear model, GLM, procedure and Tukey-Kramer least means separation test (SAS software v. 9.2, SAS Institute, Cary, NC, USA)

Do different components of methoprene and synergized pyrethrin aerosol application effect egg hatch?

Concrete Petri dishes were prepared as previously described. Five (1 cm X 1 cm) pieces of double sided sticky tape (J. V. Converting, Inc. Co., Fairless Hills, PA, USA) were placed in the center of the Petri dish. The pieces of tape were arranged with one in the middle of the Petri dish two directly above and two directly below the center piece; none of the pieces of tape were touching each other.

To obtain eggs for experiment, three groups of 100 adult *T. castaneum* were removed from established laboratory colony jars. Each group of adults was placed for 24 hrs in a 473 ml glass canning jar (Jarden Home Brands, Daleville, IN, USA) with 250 g of flour, previously sieved through a size 60-mesh sieve. Eggs from one jar were then removed from the flour using a size 60-mesh sieve; the collected eggs were placed in a

glass Petri dish. An aspirator was used to collect *T. castaneum* eggs in groups of ten; each group of 10 was placed on top of a piece of tape so that the eggs would not move around the dish.

Each concrete Petri dish was treated with either Isopar M; methoprene andIsopar M; synergized pyrethrin and Isopar M; methoprene, synergized pyrethrin, and Isopar M; or received no aerosol applications (untreated control) separately in larger plastic boxes as described above. The insecticide calculations, application, and handling of the Petri dishes after the aerosol application were as described above. The Petri dishes were observed daily for one week to evaluate percentage egg hatch. Since neonates tend to eat their egg shells after they have hatched, successful egg hatch was defined as the number of neonate larvae observed in the Petri dish which had fully escaped from the egg. Preliminary research showed that larvae were able to crawl across the adhesive tape without becoming stuck. This procedure was repeated twice with three replicates per block, resulting in six replicates total for each treatment. Percentage of egg hatch was calculated for each Petri dish, replicate, and analyzed using a GLM procedure and Tukey-Kramer least means separation test (SAS software v. 9.2, SAS Institute, Cary, NC, USA).

What is the effect of flour residues on the efficacy of methoprene and synergized pyrethrin aerosol applications against egg?

Concrete Petri dishes were made as described above but one (1 cm X 1 cm) piece of double-sided sticky tape was placed in the center of the dish. Eggs that were used in experiment were collected as described above. Ten eggs were added to each Petri dish. The dishes were sprayed with Isopar M; methoprene and Isopar M; synergized

pyrethrin and Isopar M; methoprene, synergized pyrethrin, and Isopar M; or no aerosol applications (untreated controls). The insecticide calculations and handling of the Petri dishes was the same as described above. In order to simulate different levels of sanitation, one of three amounts of flour (0.01, 1, and 5 g) was placed on the surface of the concrete dish, covering the eggs, after the aerosol application. The levels of flour represent a high level of sanitation (a flour dusting, 0.01 g), low sanitation (flour which completely covers the surface, 5 g), and an intermediate sanitation level between the two extremes (partially covers the surface, 1 g). Ten dishes were setup for each treatment combination and sanitation level and the experiment was repeated twice for a total of 20 replicates. The Petri dishes were held for 5 weeks at 27.46±0.01^{0}C, 59.45±0.05 % RH, and 16:8, light/dark photoperiod in a growth chamber (Model CTH-811, Percival Scientific Inc., Perry, Iowa, USA) and then the number of living developmental stages (larvae, pupae, and/or adults) present was determined. Data was analyzed using the GLM procedure and Tukey-Kramer least means separation test (SAS software v. 9.2, SAS Institute, Cary, NC, USA).

Results

Do different components of methoprene and synergized pyrethrin aerosol application effect adult survival and progeny production?

All *T. castaneum* adults exposed to treatments containing synergized pyrethrin suffered from knockdown 24 hr after treatment, but no knockdown was observed in the treatments which did not contain synergized pyrethrin. Adult mortality after 15 days was not significantly different among any of the treatments (Fig. 4.1) (F=0.20; d.f.=4, 287;

P=0.9358), with both sex and the interaction between sex and treatment both being not significant (F=0.00; d.f.=1, 287; P=0.9894, F=0.20; d.f.=4, 287; P=0.9358, respectively).

The average total number of living F1 progeny (adults, pupae, and larvae) after 5 weeks was not significantly different for any of the treatments (F=0.63; d.f. =4, 143; P=0.6441) (Fig. 4.2). However, the percentage of progeny with physical deformities was significantly different among the treatments (F=3.60; d.f.=4, 143; P=0.0079). The differences among treatments resulted from the Isopar M treatment having fewer deformities (16±2%) than either of the two treatments that contained methoprene (methoprene and Isopar M (24±3%) and methoprene, synergized pyrethrin, and Isopar M (24±4%)). The other two treatments were not significantly different from each other or any of the previously mentioned treatments: control (15±2%) and synergized pyrethrin and Isopar M (20±3%).

Do different components of methoprene and synergized pyrethrin aerosol application effect egg hatch?

Differences in egg hatch among the treatments were observed (F=94.22; d.f.=4, 412; P<0.0001) (Fig. 4.3). Day of hatch (F=125.80; d.f. =2, 412; P<0.0001), interaction between treatment and day of hatch (F=24.71; d.f.=8, 412; P<0.0001), and interaction between block and day of hatch (F=12.02; d.f.=2,4 12; P<0.0001) were also significant. However, interactions between block and treatment (F=1.66; d.f.=4, 412; P=0.1592) and block, treatment, and day of hatch (F=1.82; d.f.=8, 412; P=0.0711) were not significant. Comparing egg hatch among the treatments, significantly more eggs hatched in the untreated control and in the Isopar M treatment than eggs that were exposed to

methoprene and Isopar M; synergized pyrethrin and Isopar M; and the combination of methoprene, synergized pyrethrin, and Isopar M.

What is the effect of flour residues on the efficacy of methoprene and synergized pyrethrin aerosol applications against egg?

The average number of adults that were observed five weeks after eggs were exposed to either Isopar M, methoprene and Isopar M; synergized pyrethrin and Isopar M; methoprene, synergized pyrethrin, and Isopar M; or not exposed to aerosols (control) and then had a level of flour (0.01, 1, or 5 g) placed on the eggs was significantly different among treatments in the overall model ($F=69.82$; d.f.=14, 349; $P<0.0001$). Insecticide treatment, sanitation, and the interaction between the insecticide treatment and sanitation level were all significant ($F=111.93$; d.f.=4, 359; $P<0.0001$, $F=110.01$; d.f.=2, 361; $p<0.0001$, $F=40.14$; d.f.=8, 355; $P<0.0001$; respectively). Among the insecticide treatments, the average number of adults was highest in the controls and significantly greater than the Isopar M treatment which in turn was higher and significantly different from the other insecticide treatments. The mean number of adults in the methoprene, synergized pyrethrin, and combination of methoprene, synergized pyrethrin, and Isopar M was essentially zero for all three of these groups and significantly different from the eggs exposed to Isopar M or untreated controls.

The number of adults was significantly different among the levels of flour (Tukey-Kramer test, $P<0.05$), with no adults observed in the 0.01 g (Fig 4.3A) flour level treatment, higher numbers observed in the 1 g (Fig. 4.2B) flour level treatment, and the highest numbers in the 5 g (Fig. 4.3C) flour level treatment. The significant interaction between insecticide treatment and flour depth resulted from the significant differences

between the number of adults between the control and Isopar M treatment groups and that each flour depth has an overall significantly different number of adults from the other flour depths.

The full GLM model (SAS software v. 9.2, SAS Institute, Cary, NC, USA) for the average number of immatures observed five weeks after direct exposure to Isopar M; methoprene and Isopar M, synergized pyrethrin and Isopar M; methoprene, synergized pyrethrin, and Isopar M; or not exposed (flour control) and one of three levels (0.01, 1, or 5 g) of flour added to each treatment combination showed significant differences ($F=27.22$; d.f.=14, 349; $P<0.0001$) in the average number of immatures observed between treatments and flour depths. The average number of immatures observed was significantly different among insecticide treatment ($F=48.86$; d.f.=4, 359; $P<0.0001$) and sanitation level ($F=50.22$; d.f.=2, 361; $P<0.0001$) with a significant interaction between treatment and sanitation ($F=12.64$; d.f.=8, 355; $P<0.0001$).

Eggs that were exposed to methoprene, synergized pyrethrin, and Isopar M had significantly greater number of immatures than those that were exposed to all other treatment combinations; eggs exposed to synergized pyrethrin and Isopar M had the lowest number of immature developmental stages. However, this was not significantly different from eggs that were exposed to Isopar M; methoprene, synergized pyrethrin, and Isopar M; or untreated (controls). The mean number of immatures in eggs that were covered with 0.01 g (Fig. 4.3A) and 5 g (Fig. 4.3C) of flour were significantly lower than eggs that were covered in 1 g (Fig. 4.3B) of flour. This is likely due to the fact that the intermediate flour level, 1 g, had a mix of developmental stages but the 0.01 g only had larvae and the 5 g had mostly adults and few larvae. The significant interaction

between treatment and flour depth is potentially due to the greater number of immatures in the1 g flour level and the fact that the number of immatures from eggs treated with methoprene and Isopar M was significantly different from all other treatment combination at the 1 g and 5 g flour levels.

Discussion

Application of synergized pyrethrin, either alone or in combination with methoprene, resulted in 100% knockdown of *T. castaneum* adults, but these individuals recovered and ultimately there was no difference among any of the insecticide treatments or the control. The egg hatch was reduced by methoprene and Isopar M but further reduced in synergized pyrethrin and Isopar M in comparison to the unexposed eggs (control) and those exposed to Isopar M alone. The addition of methoprene to synergize pyrethrin and Isopar M did not further reduce egg hatch. Adding flour to the eggs and allowing for 5 weeks of development showed the potential effect of sanitation and efficacy of the insecticides. In the 0.01 g flour depth no adults were able to develop and the eggs which did develop were very tiny larvae. As the flour depth increased, from 0.01 g to 1 g and to 5 g, the number of adults increased but no or very few adults were produced in the two treatment groups containing methoprene. Immature development however was highest at the 1 g flour depth and in the methoprene and Isopar M treatment groups.

The rate of recovery of *T. castaneum* adults exposed to synergized pyrethrin in Isopar M or in combination with methoprene is higher than previously reported (Arthur and Campbell, 2007; Arthur 2008). However, Arthur and Campbell (2007) also reported the potential for *T. castaneum* to recover from knockdown effects from the exposure to

synergized pyrethrin when placed in clean flour after the exposure. Conversely, Arthur (2008) reported a high rate of mortality of *T. castaneum* regardless of the presence of a flour patch during an aerosol application of synergized pyrethrin.

The presence of the flour patch with *T. castaneum* adults simulates the condition of a flour patch which was not removed during routine sanitation or low sanitation conditions; the flour patch can serve as a shelter in which the insect could escape or hide from the insecticide applications. Whereas our study simulates *T. castaneum* adults out in the open, directly exposed to aerosol application, and then moving into a hidden area where they come in contact with other individuals in a flour patch and reproduce in the residual patch; the expectation is that there would be higher mortality under direct exposure and then movement into the flour patch. However, previous work has shown that methoprene is easily transferred to surfaces (Chapters 2 and 3) and it is possible that synergized pyrethrin can also be moved but this has not been evaluated.

The fact that there were no differences in the total number of living F1 progeny suggests that neither vertical nor horizontal transfer of methoprene occurred either by contact with the *T. castaneum* parents or by moving through the flour which could potential have some insecticide residues from the *T. castaneum* adults which were directly treated with insecticides. Although horizontal transfer of methoprene has been previously reported in *T. castaneum* (Chapters 2 and 3) it is not surprising that is wasn't detected here since there were no significant differences in parent mortality of the different treatment combinations.

Egg mortality after being treated with the carrier Isopar M or untreated (control) was less than 25%. This matches previous studies where *T. castaneum* egg mortality

found that mortality ranged from 10% (Sokoloff, 1974) to 25% (Brijwani et al., 2012). Eggs that were treated with methoprene and Isopar M had a lower hatch rate than those in the control or the Isopar M but individuals exposed to either synergized pyrethrin and Isopar M or methoprene, synergized pyrethrin, and Isopar M had the lowest successful hatch rate. It is possible that extending the egg hatch out past 6 days would increase the successful egg hatch but neonate larvae readily consume their egg shells and on day seven were observed cannibalizing neonate larvae and eggs which either had not hatched or were in the process of hatching. Cannibalization has been previously reported (Park et al., 1965).

The three different flour residues applied to eggs post-aerosol application were designed to simulate different levels of sanitation. The treatment representing a high level of sanitation, which corresponded to a low amount of flour, 0.01 g, was not sufficient for the beetles to complete development. No adults were observed and the surviving larvae were much smaller than in the other treatments. Thus there was little potential to detect effects of the insecticides. The fact that the eggs were exposed to methoprene and Isopar M, Isopar M alone, and untreated control all had greater than 40% egg hatch at day 6 supports the hypothesis that the small amount of flour was insufficient for complete development in the 0.01 g flour Petri dish. It is also possible that more larvae successfully hatched from treated eggs but due to previously described cannibalism (Park et al., 1965) there was no evidence of their presence (preliminary data demonstrated that the larvae at this flour level would cannibalize each other). High sanitation levels (low flour residues) improved the efficacy of synergized pyrethrin and pyrethroid aerosol applications (Toews et al., 2010) and heat treatments (Brijwani et al.,

2012). However, our results suggest that in low amounts of flour residues *T. castaneum* development is not likely to occur and therefore effect of insecticide effects cannot be detected.

The mixture of mostly larvae and pupae along with a small number of adults in the 1 g flour Petri dishes regardless of treatment; and a majority of adults in control and Isopar M treatments, many larvae in the methoprene and Isopar M treatments, and a higher number of adults than immatures in the treatments which included synergized pyrethrin suggest that the insecticide treatments have a delayed effect on development. This observation has been reported previously for *Plodia interpunctella* eggs and larvae exposed to methoprene (Jenson et al., 2009).This delay in development could potentially result in population effects which will suppress insect population growth.

The low numbers of adults in the treatments receiving an insecticide application indicate a high insecticide efficacy on insect development. However, there were a greater number of immature developmental stages in the 1 g and 5 g flour Petri dishes. While these differences were not significantly different the presence of insects suggests a potential for incurring some economic losses due to the presence of live insects and cast skins (Baur, 1984). However, this was only one spray application and it is likely that with repeated aerosol applications there would be a greater effect.

In conclusion, flour residues due to different levels of sanitation play a role in the efficacy of insecticides. When sanitation is poor methoprene, synergized pyrethrin, and the combination of methoprene and synergized pyrethrin suppress egg hatch and the subsequent development to adulthood. However, as the depth of flour increases more individuals successfully emerge as adults, but insecticides became more effective.

However, as flour depth increases it is more likely that the individuals can readily escape from the insecticides. The components of methoprene and synergized pyrethrin, alone or in combination, appear to have little effect on the mortality of adults or on subsequent F1 progeny. However, this experiment only used one aerosol application and it is possible that with multiple spray applications there are cumulative effects on individuals. These cumulative effects could result in the reduction if beetle populations.

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Figures

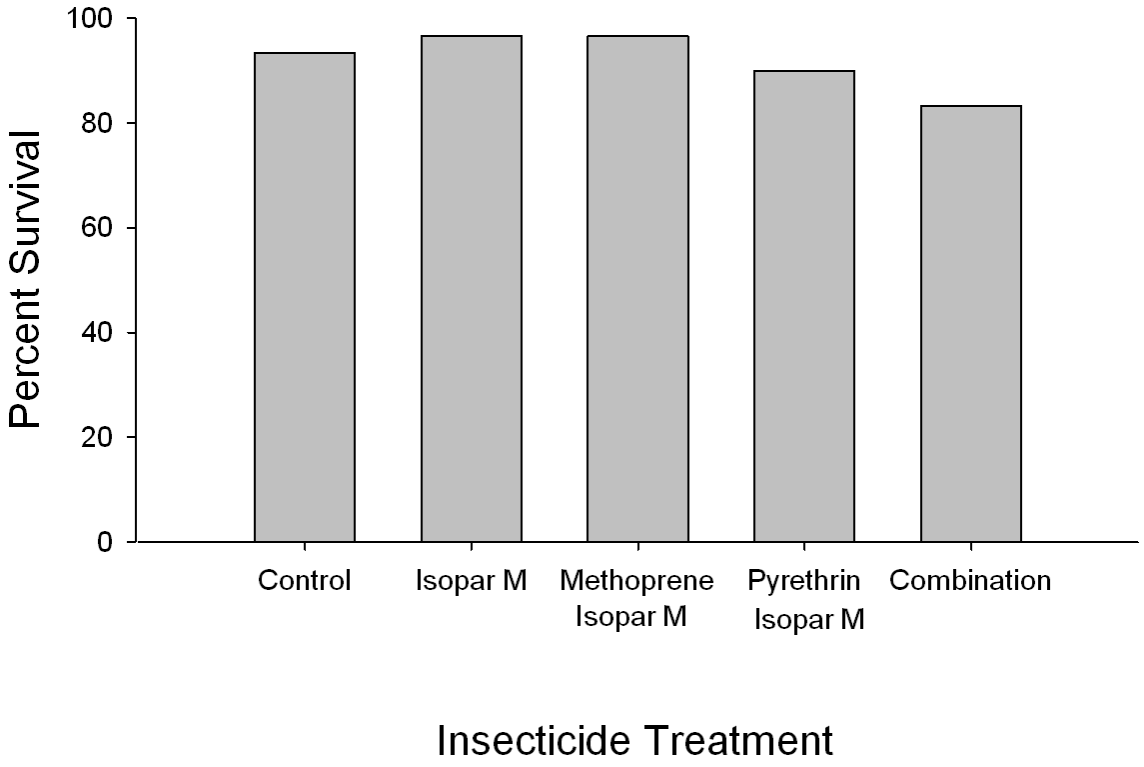

Figure 4.1 The percentage of adults that were alive a week after exposure to aerosol applications of Isopar M; methoprene and Isopar M; synergized pyrethrin and Isopar M; methoprene, synergized pyrethrin, and Isopar M; or not exposed (control). There were no significant differences in mortality means based on Tukey-Kramer least square means for multiple comparisons ($P>0.05$).

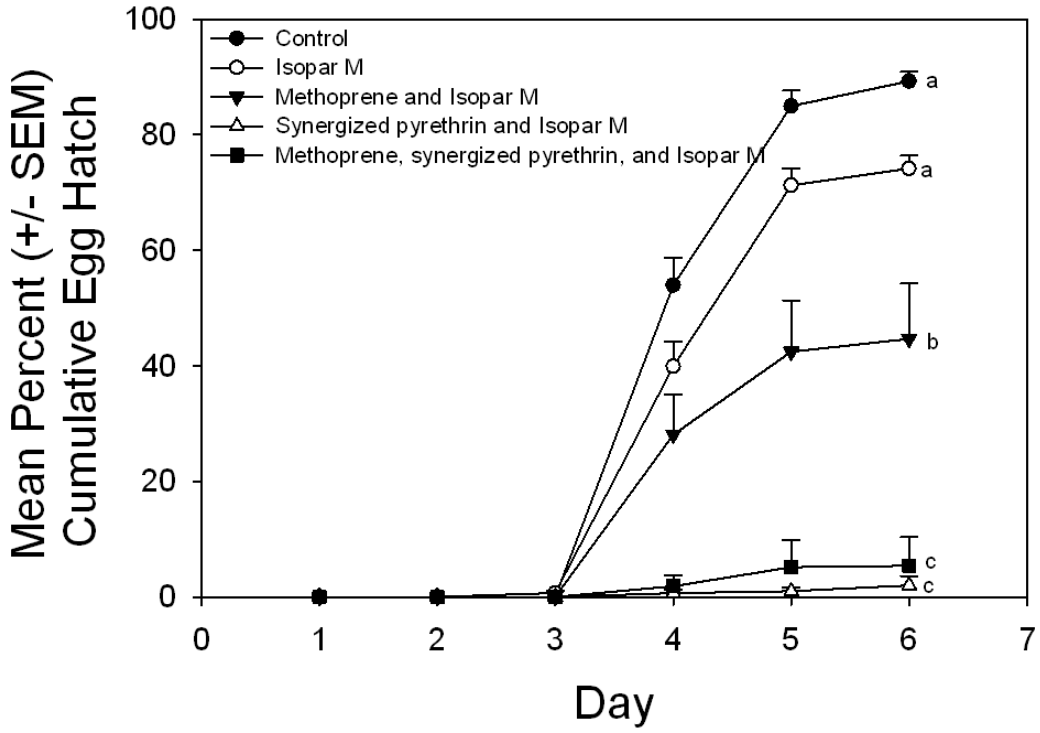

Figure 4.2 The percentage (mean±SEM) cumulative egg hatch of 24 hour *T. castaneum* eggs which received an aerosol application of Isopar M; methoprene and Isopar M; synergized pyrethrin and Isopar M; methoprene, synergized pyrethrin, and Isopar M; or no aerosol applications (control). Same lowercase letters next the treatment on day 6 indicate no significant differences in means according to Tukey-Kramer least square means comparison test (P>0.05).

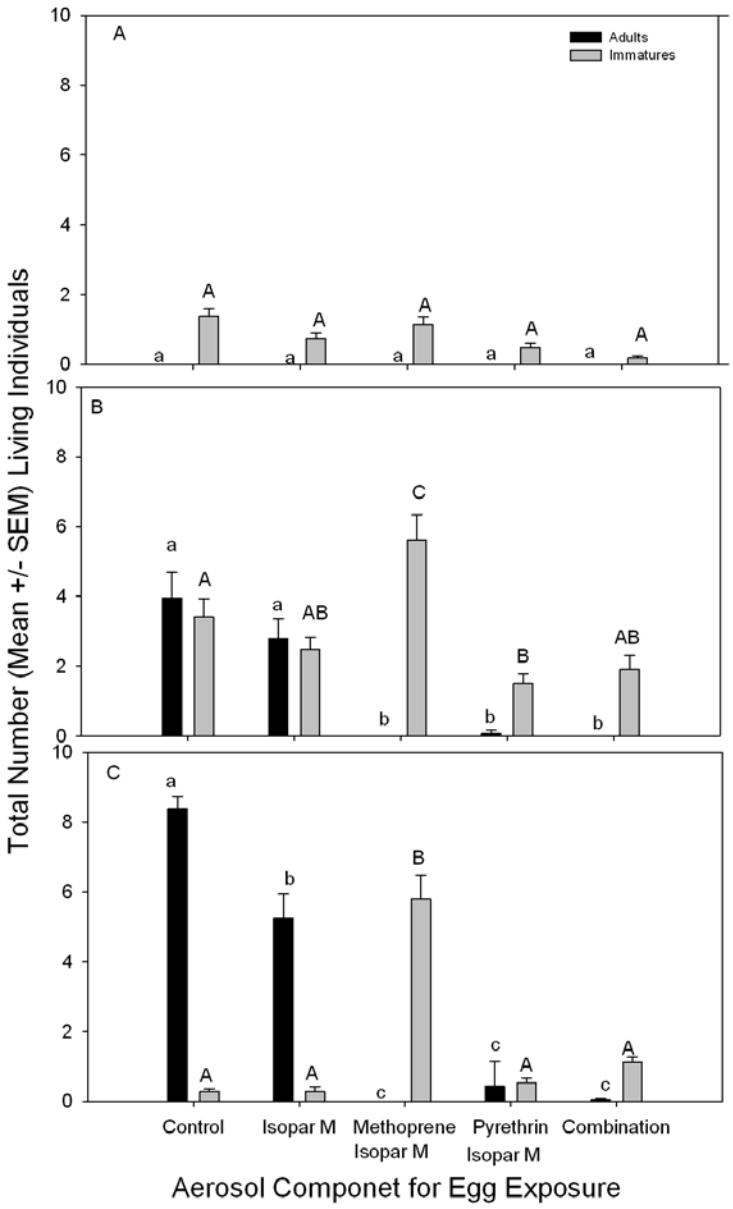

Figure 4.3 The number (mean ± SEM) of *T. castaneum* adults or immatures present five weeks after an aerosol application of Isopar M; methoprene and Isopar M; synergized pyrethrin and Isopar M; methoprene, synergized pyrethrin, and Isopar M; or no aerosol application (control) and an amount of flour (0.01 g (A), 1 g (B), or 5 g (C)) was added post-aerosol application. Same lowercase (adult) or uppercase (immature) letters above bars indicate no significant differences among insecticide treatment means within a flour depth according to least square means test with a Tukey-Kramer multiple comparisons adjustment (P>0.05).

Chapter 5 - The Effect of Methoprene and Synergized Pyrethrin Aerosol Applications on *Tribolium castaneum* (Herbst) Populations

Abstract

Experiments were performed in order to investigate the effects of horizontal transfer of methoprene, sanitation, and multiple applications of methoprene and synergized pyrethrin on *Tribolium castaneum* populations. The final subsample of total number of living individuals from colonies which received adults that were treated with Isopar M or no adults (control) was significantly greater than those that received synergized pyrethrin ($P<0.05$) treated adults suggesting that synergized pyrethrin can reduce *T. castaneum* populations. The total number of living individual and instantaneous rate of increase (r_i) in the subsamples and the final count had no significant differences in established populations with a hidden refugia after multiple applications of methoprene and synergized pyrethrin compared to those which received no insecticide applications ($P>0.05$). The nested sanitation levels inside the two treatment groups also had no effects ($P>0.05$) suggesting that populations in hidden refugia can persist even with multiple applications of methoprene and synergized pyrethrin and sanitation measures. Populations with an accessible hidden refugia that were exposed to the combination of methoprene and synergized pyrethrin has significantly lower number of living adults and r_i than populations that were exposed to synergized pyrethrin alone ($P<0.0001$). Additionally, populations which receive one, two, or three aerosol applications had the same number of living adults and r_i but were significantly different from populations

which received four aerosol applications. This suggests that the combinations of methoprene and synergized pyrethrin could be more effective than synergized pyrethrin when multiple aerosol applications are performed and cumulative effects on populations could occur.

Introduction

Tribolium castaneum (Herbst), the red flour beetle, is a cosmopolitan pest in flour mills, warehouses, and other post-harvest food processing structures. The presence of *T. castaneum* in post-harvest food processing structures causes economic losses due to contamination of products and the costs involved with reducing insect populations. *T. castaneum* populations can be difficult to control since they exploit hidden areas such as cracks and crevices, inside equipment, and inside building structures (Campbell et al., 2004; Toews et al., 2005).

One component of *T castaneum* pest management is routine aerosol insecticide applications (Peckman and Arthur, 2006). The droplets in aerosol applications provide good coverage of exposed surfaces, but can't penetrate into hidden areas (Peckman and Arthur, 2006; Arthur, 2008) where the majority of *T. castaneum* populations are located. Aerosol applications would therefore appear to be an inefficient method of treating *T. castaneum* populations. However, regular aerosol applications of methoprene and synergized pyrethrin as part of an integrated pest management program has been reported to suppress fluctuations in trap captures within a flour mill (Campbell et al., 2010ab). This suggests that the cumulative effects of treating the small part of the population exposed to the treatment or movement of insecticide into

these hidden areas where most of the population occurs may be effecting population growth.

The combination of methoprene and synergized pyrethrin is widely used in aerosol insecticide applications since methoprene, which is an insect growth regulator, targets immatures as they transition from one developmental stage to another (Beckage, 1998; Henrick, 2007) and synergized pyrethrin results in knockdown of insects and direct kill (Jensen et al., 2010). The methoprene component of these applications can also have good persistence on exposed surface (Sutton et al. 2011) which can increase long term efficacy. The immediate effects of synergized pyrethrin and methoprene aerosol applications on different *T. castaneum* developmental stages have been evaluated (Arthur, 2008; Toews et al., 2010; Chapters 2, 3, and 4). However, the effects of these insecticide treatments on *T. castaneum* populations have not been evaluated. While the results from individual toxicological studies provide an indication of the insecticide efficacy, this information may not reflect efficacy on population (Stark and Banks 2003; Toews et al., 2005).

Insecticides applied to exposed surfaces may migrate into hidden areas using a variety of mechanisms. Previous work demonstrates that horizontal transfer of methoprene from a treated individual to another individual can occur and that contact and contamination of a food-source (flour) are the most likely mechanisms (Chapter 2 and 3). However, the effects of these treatments are highly variable and mortality rates are often low. It has also been shown that although synergized pyrethrin can have an initially high knockdown effect, these individuals can recover and not exhibit a loss in progeny production (Chapter 4). These findings also suggest there is limited effect from

single applications, but in real world applications, food facilities typically apply aerosol insecticides at regular intervals. These repeated applications may increase the effect of these treatments on population growth and generate the observed results in commercial facilities (Campbell and Arbogast, 2004).

In this series of experiments, the effects of multiple aerosol applications on populations in hidden untreated refugia were evaluated under three different scenarios: controlled immigration of methoprene and synergized pyrethrin treated adults into a *T. castaneum* population, multiple aerosol applications on an established *T. castaneum* population were individuals could move in and out of an untreated refugia, and multiple aerosol applications on a recently established *T. castaneum* populations where individuals could freely move in and out of an untreated refugia.

Materials and Methods

A recently collected *T. castaneum* colony (Romero et al., 2010) was used in the following experiments. Insect cultures were maintained at 28.3±0.0^{o}C, 46.3±0.1% RH and 14/10 (day/night) photoperiod and rearing procedures were described in detail in Chapter 2. Adult developmental stages used in experiments were collected six weeks after the initial inoculation with adults. Specimens used in this research were deposited in the Kansas State University Museum of Entomological and Prairie Arthropod Research (KSU-MEPAR) under voucher number 226.

Does immigration of methoprene and synergized pyrethrin treated adults affect populations?

Effect of immigration of aerosol insecticide treated adults on an established population was evaluated by adding treated individuals every two weeks to jars

containing a *T. castaneum* population in flour. Experimental populations of *T. castaneum* were created by adding 5 male and 5 female adults to 200 g of flour in a glass jar, and holding these jars for 3 months to allow population levels to grow. Samples were collected from jars immediately prior to starting to add treated insects and then at two week intervals for four months. Jars were sampled by mixing the jars to evenly distribute the developmental stages, removing 1 g of flour with a plastic spoon from the center of the jar, counting the number of individuals in each developmental stage, assessing if individuals exhibited deformities, and the number of alive and dead individuals; samples were not placed back into the colony jars after they were counted. At the end of the experiment a larger 2 g subsample of the flour was taken in the same manner as previously described and the same data was recorded.

Insecticide treated adults for addition to jars were created using the following protocol. Adult *T. castaneum* were sexed by the presence, male, or absence, female, of the setiferous patch (Hinton, 1942). Approximately 50 males and 50 females were placed on separate 90 x 15 mm (62 cm^2) Petri dishes with a concrete bottom (Rockkite®, Hartline Products, Co., Inc., Cleveland, OH, USA). The Petri dishes were then placed in a plastic rectangular box with a layer of concrete on the bottom (length 56.5±0.2 cm, width 34.6±0.3 cm, and height after addition of concrete 15.5±0.1 cm) for the simulated aerosol applications. In separate boxes for each treatment, the different components of a combination pyrethrin and methoprene application were applied: the carrier Isopar M (Entech Systems, Kenner, LA, USA); methoprene (Diacon® II, Central Sciences International, West Schaumberg, IL, USA) and Isopar M; synergized pyrethrin (Entech Fog-10, Entech Systems, Kenner, LA, USA) and Isopar M; or the combination

of methoprene, synergized pyrethrin, and Isopar M. The amount of insecticide component applied to each box was the amount estimated to settle on a surface area of the same size within a food facility during an aerosol application, assuming 75% insecticide deposition on the floor and using the volume of a specific floor of a flour mill.

The specific insecticide calculations are reported in Chapter 4, but this translates into 0.0045 mg active ingredient (a.i.) of methoprene in Isopar M and 0.02 mg a.i. of synergized pyrethrin g/cm^2 in Isopar M; the same amount a.i. was present in the combination of methoprene, synergized pyrethin, and Isopar M. Insecticide was applied evenly to the concrete surface of the box, including the dishes containing the insects, using an artist's air-brush (Badger Air-Brush Company, Franklin Park, IL, USA). The boxes were allowed to vent the insecticide for one hour. The insects were placed in new Petri dishes and frozen, for 24 hrs at 54.0±0.2% RH and -16.8±0.0^{o}C, in the freezer section of a Hotpoint household freezer/refrigerator (General Electric Co., Louisville, KY, USA). The adults were frozen to reduce degradation of insecticides (Henrick, 2007) and to standardize treatment conditions (Chapters 2 and 3) since synergized pyrethrin result in adult knockdown or mortality and methoprene does not. This process was repeated for every new addition of treated insects. Eight new additions were performed.

Five males and five females from each treatment group were placed in each respective designated experimental jar with established populations. The jar was gently agitated for 1 min to simulate movement of the treated individuals into a hidden area. Control jars were sampled and agitated in the same manner as the insecticide component jars, but did not receive any treated adults since previously experiments

showed that the water controls had little or no effect on the insect development (Chapters 2 and 3). There were nine jars for each insecticide treatment and control.

The resulting data (total number of living individuals) from the 1 g samples was analyzed with GLIMMIX (SAS v. 9.2 software, SAS Institute, Cary, NC, USA) using a repeated measures model with each subsample date as the time point; if significant differences between treatments (control; Isopar M; methoprene and Isopar M; synergized pyrethrin and Isopar M; or the combination of methoprene, synergized pyrethrin, and Isopar M) were found then a means separation procedure such as Tukey-Kramer was used to determine which treatments were significantly different. The instantaneous rate of increase (r_i) was calculated for each treatment group using the equation [$r_i = ((\ln (N_f/N_0)) / \Delta T$); where N_f = current population size, N_0 = the founding population size, and ΔT is the change in time] (Stark and Banks, 2003). The value of the r_i determines if the population is stable (r_i is zero or approaching zero), declining (r_i is negative), or increasing (r_i is approaching or equal to one) (Stark and Banks, 2003). The r_i from the subsamples was analyzed with the same repeated measures analysis described above. The final 2 g subsample was analyzed using the General Linear Models (GLM) procedure (SAS v 9.2 software, SAS Institute, Cary, NC, USA) for both the total number of individuals and the r_i; significant differences amount treatments were determined using the GLM Tukey means separation test.

Do multiple methoprene and synergized pyrethrin aerosol applicait0ons and sanitation measures affect populations?

Populations of *T. castaneum* were exposed to repeated insecticide applications in arenas with a refugial area containing flour that was not directly treated and beetles

were allowed to move back and forth between treated and untreated areas. The arenas consisted of plastic boxes with a layer of concrete patch material as described above. In addition, a 90 x 15 mm (62 cm^2) Petri dish was inserted into the concrete patch material before it was set; this dish was located approximately in the middle of the width of the box and 15 cm from the left side and 34 cm from the right side. The Petri dish served as the refugia and contained 15.0±0.0 g flour and was covered with a metal plate (7.5 x 7.5 x 0.1 cm) elevated 0.2 cm above the surface by two metal spacers (1.7 x 1.7 x 0.1 cm each) stacked on top of each other and placed in the corners of the metal plate. Two paper strips (2 cm by 2 cm) were placed in the flour and leaned on the lip of the Petri dish for beetles to walk on when moving in and out of the Petri dish. Experimental populations were started by adding 25 males and 25 females to each plastic container with flour. One month after establishing the population the insecticide treatments were initiated.

The amount of insecticide applied to each arena was discussed in Chapter 4. This resulted in each container which received an aerosol application receiving 1.4 mg a.i. for methoprene and 5.0 mg of a.i. for synergized pyrethrin in a total volume of 0.5 ml of the mixture synergized pyrethrin in Isopar M with methoprene added. Prior to the aerosol applications, the number of individuals outside the refugia was counted and all but five were returned to the Petri dish refugia and the refugia covered with the metal plate and the spacers were placed on top of the metal plate. Covering the refugia sheltered the flour and insects in the Petri dish from direct spray during the aerosol application. The five individuals simulated the small percentage of a population that might be directly exposed during treatment. The insecticide was applied using the

artist's airbrush technique previously described and there was a 1 hr venting period after treatment before the boxes were covered. Twenty-four hrs after treatment the spacers were returned under the metal plate so that insects could enter and leave the refugia. The control containers did not receive an aerosol application, but were covered with metal plate for an equivalent period of time.

Six replicate arenas received no aerosol application (control) and six received aerosol applications of a combination of methoprene, synergized pyrethrin, and Isopar M every two weeks for a total of eight applications. Nested within each treatment and control treatments were two levels of simulated sanitation. Treatments or controls with spillage received a 1.0 ±0.0 g dusting of flour (referred to as dusting or flour dusting) after each aerosol treatment and this material was brushed up with a hand-held broom and removed before the next insecticide application. Treatments or controls with no spillage (referred to as clean) did not have any additional flour added. The spillage treatment was hypothesized to encourage beetles to spend more time out of the refugia and potentially result in increased exposure to treated surfaces and the insecticide may be absorbed into the flour which could also increase exposure. The flour dusting was placed on the concrete surface of treatments designated for dusting 24 hours after the first aerosol application. The flour dusting was removed from the surface prior to aerosol application. This process was repeated for each aerosol application after the first one. The insecticide and nested sanitation levels resulted in four treatment groups: control with no flour dusting, control with flour dusting, methoprene and synergized pyrethrin without flour dusting, and methoprene and synergized pyrethrin with flour dusting.

The arenas were sampled three times during experiment: 10, 15, and 20 weeks. Samples were collected by mixing the contents of the Petri-dish, removing a subsample (0.8±0.1 g) of flour, and counting and recording the number of living and dead individuals of each developmental stage in the sample. Subsamples were not returned to the Petri dish after counting. At the end of the experiment, after the last subsample was taken all of the remaining individuals, dead and alive, of all developmental stages were counted as well as the number deformed individuals. The r_i was calculated for each subsample and the final population counts. The subsamples and final counts and r_i will be analyzed as described above. The number of living individuals and r_i in insecticide treatments were compared to the controls; the same comparison were made for the two different sanitation levels.

Do multiple methoprene and synergized pyrethrin aerosol applications affect populations?

This experiment had the same as objective as experiment 2 but was improved to simulate more real world scenarios and to gain a better understanding of the effect of multiple aerosol applications of methoprene, synergized pyrethrin, and Isopar M. The improvements included: a smaller arena was used in order to increase the replication number, the refugia was left open during aerosol applications which allowed *T. castaneum* individuals to escape the effects of direct aerosol exposure and allow for the movement of the insecticides into the hidden area and thereby secondarily exposing previously unexposed individuals, and the dishes were placed in a room for aerosol application which required the use of a handheld applicator which wascloser to how aerosol applications would occur in a flour mill.

The population arenas were made with a plastic Petri dish (25 X 150 mm; 1766.25 cm^2 area of bottom dish) containing a layer of concrete in the bottom, prepared as described above, and the inner walls coated with liquid Teflon (Fluon AD-1, Northern Products, Inc., Woodsocket, RI, USA) to confine the beetles to the arenas. Refugia for each arena were created by placing 3.5 g of flour in a 7.39 ml glass screw thread vial (17 x 60 mm; Kimble Chase Life Science and Research Products, Vineland, NJ, USA) and sealing the vial with a plastic lid. The plastic lid had a 7/64 inch (0.28 cm) opening created by a drill which allowed for beetles to move in and out of the vial. The front of the plastic cap was sanded with a piece of 100 grit aluminum oxide resin/gum sandpaper and a 3/8 inch (0.95 cm) Dremel Multipro (Model 395T6, Racine, WI, USA) was used to sand the interior side of the plastic cap to facilitate climbing by the beetles. Preliminary studies showed that beetles could enter and exit the vial after the above modifications to the vial cap were performed. *T. castaneum* populations were established by placing two virgin males and females (founders) inside the glass vial in the Petri dish; insecticide applications started two weeks after the founders were added to glass vials. Dishes were held in an incubator at 31.0±0.01^{o}C and 60.0±0.07% RH when they were not being treated with insecticides.

Petri dishes were treated with aerosol applications of insecticides by placing them on the floor of sheds (58.9 m long X 25.7 m height X 28.3 m width) lined with plastic. To account for potential variation in insecticide deposition six Petri dishes were placed in each shed, three on the left side and three on the right side of the shed (Fig. A.1); the lids were placed face up next to the dish. Insecticides that were used were the same commercial products as previously used (Chapter 2, 3, and 4 as well as

above). The labeled rate of synergized pyrethrin (29.6 ml / 28.3 m^3) in Isopar M or combination of methoprene (3 ml / 283.2 m^3) added to the labeled rate of synergized pyrethrin in Isopar M was used; this translates to 450 mg a.i. of synergized pyrethrin in Isopar M applied to each shed and 388.8 mg of a.i. of methoprene was added to the synergized pyrethrin and Isopar M to sheds that received the combination of methoprene, synergized pyrethrin, Isopar M. Assuming 75% deposition to the surface of the floor, each dish that was in a shed treated with synergized pyrethrin in Isopar M would receive 3.5 mg a.i. and dishes that received the combination would also receive 3.1 mg ai. A hand held aerosol applicator (FogMaster Jr, Model 533010CA, The Fogmaster Corp., Deerfield Beach, FL, USA) was used to treat the sheds. Insecticides were applied by walking down the middle of the shed between the two rows of Petri dishes and moving the aerosol applicator from left to right. In order to determine the time needed to apply the appropriate amount of insecticide the aerosol applicator was calibrated using water. Then the water calibration mass/time of release ratio was used to calculate the time needed for correct insecticide release.

After a 12 hr exposure time and additional one hr ventilation period the dishes were removed and returned to the incubator. Treatment dishes received 1, 2, 3, or 4 aerosol applications at two week intervals; the control Petri dishes were not sprayed and were held in the incubators. There were four replicate aerosol applications for each treatment. The controls and insecticide treatments which received 1-3 insecticide applications had 48 dishes total, but the insecticide treatments which received 4 aerosol applications had 96 dishes (dishes originally slated for more aerosol applications were combined into the four application group due to status of the insect populations). At the

end of the experimental period, the total number of living, dead, and deformed adult *T. castaneum* in the arena and the vial were counted. Six Petri dishes from each insecticide and number of aerosol sprays were randomly selected to count the total number of progeny (pupae, larvae, and eggs).

The r_i was calculated, using total adult counts, for each Petri dish. The total count of living adults in the synergized pyrethrin and Isopar m and combination of methoprene, synergized pyrethrin, and Isopar M and the r_i were calculated and analyzed with GLM as described above. The GLM model included the number of aerosol applications and the insecticide treatments to determine their effect on populations. The data from the controls was not analyzed as it did not follow the same treatment analysis structure as the Petri dishes in the insecticide treatments. The progeny subsample counts could not be analyzed due to the sparseness of data.

Results

Does immigration of methoprene and synergized pyrethrin treated adults affect populations?

The total number of living individuals in subsamples had significant differences among the treatments (F=2.92; d.f.=4, 240; P=0.0221) (Fig. 5.1A). The control was significantly lower than the combination of methoprene and synergized pyrethrin but was the same as all other treatment combinations. In the overall GLIMMIX model, sample date (F=4.97; d.f.=5, 240; P=0.0002) was significant but the interaction between treatment and sample date (F=0.50; d.f.=20, 240; P=0.9640) was not significant. The drop in total individuals at the last sample date suggests that the jars were becoming over utilized and the population was ready to decline; the overutilization

of the jars could explain why the control jars had the lowest populations numbers since they would have no mortality related to insecticide treated individuals, therefore, their populations would have declined faster than the other treatments.

The instantaneous rate of increase (r_i) was not significantly different (F=2.11; d.f.=4, 240; P=0.0799) (Fig. 5.1B) between the treatments. In the overall model sample date (F=2.60; d.f.=5, 240; P=0.0258) was significant but the interaction between insecticide treatment and sample date (F=0.46; d.f.=20, 240; P=0.9775) was not significant.

At the end of the experiment (2 g subsamples), there were significant differences in the mean number of living individuals (F=4.15; d.f.=4, 37; P=0.0071) among the treatments. The control (491±44) and Isopar M (488±26) colonies were significantly greater than the synergized pyrethrin colonies (368±26). The methoprene (445±23) and combination of methoprene and synergized pyrethrin (377±24) colonies were not different from any of the other treatments.

The mean instantaneous rate of increase (r_i), calculated for the final 2 g subsample, was significantly different between the treatments. The r_i in the colonies which received Isopar M treated adult (0.0219±0.0003) and no treated adults (control) (0.0218±0.0005) was significantly greater (F=4.13; d.f.=4,37; P=0.0072) than the instantaneous rate of increase in the synergized pyrethrin and Isopar M (0.0202±0.0004) colonies which was the same as the instantaneous rate of increase in the methoprene and Isopar M (0.0214±0.0003) and combination of methoprene, synergized pyrethrin, and Isopar M (0.0204±0.0003) according to GLM and the least square means test with a Tukey-Kramer adjustment for multiple comparisons. The

methoprene and Isopar m and synergized pyrethrin and Isopar M had the same mean instantaneous rate of increase as the Isopar M and control colonies.

Do multiple methoprene and synergized pyrethrin aerosol applications and sanitation measures affect populations?

The total number of living individuals in the subsamples (Fig. 5.2A) was not significantly different by treatment ($F=0.09$; d.f.=1, 21; $P=0.7699$), sanitation ($F=0.00$; d.f.=1, 21; $P=0.9631$), or the sample date ($F=1.37$; d.f.=1, 21; $P=0.2552$). Additionally, there were no significant interactions ($P>0.05$) between the treatment, sanitation, and sample date. The instantaneous rate of increase (Fig. 5.2B) calculated was not significantly different for the treatment ($F=0.01$; d.f.=1, 21; $P=0.9312$) or the level of sanitation ($F=0.02$; d.f.=1, 21; $P=0.8868$), but was significantly different for sample date ($F=4.55$; d.f.=2, 21; $P=0.0227$). None of the interactions between the treatment, sanitation, and sample date were significant ($P>0.05$).

The count of the total number of living individuals at the end of the experiment after either no insecticide applications (control) or multiple exposures to the combination of methoprene, synergized pyrethrin, and Isopar M (nested within each treatments were a clean concrete arena or arena with a dusting of flour to represent different levels of sanitation) found no significant differences ($F=1.11$; d.f.=3, 7; $P=0.4070$) between the treatments. For the specific factors in the model, treatment ($F=0.70$; d.f.=1, 9; $P=0.4311$) (control was 235±103 individuals compared to 148±53 individuals for the combination of methoprene, synergized pyrethrin, and Isopar M), sanitation level ($F=0.27$; d.f.=1, 9; $P=0.6211$) (clean was 155±40 individuals compared to 244±127 individuals in the flour

dusted treatments), and the interaction between the treatment and sanitation level (F=2.30; d.f.=1, 9; P=0.1730) was not significant.

The instantaneous rate of increase also did not have significant differences in the overall model (F=3.42; d.f.=3, 7; P=0.0817) between the treatments. The instantaneous rate of increase in the control treatment (0.0093±0.0023) was not significantly different (F=3.11; d.f.=1, 9; P=0.01211) from the combination of methoprene, synergized pyrethrin, and synergized pyrethrin (0.0065±0.0024); the clean sanitation level (0.0076±0.0014) was not significantly different (F=3.12; d.f.=1, 9; P=0.1205) from the sanitation level which received a flour dusting (0.008492±0.0034). The interaction between the treatment and the sanitation level was not significant (F=3.17; d.f.=1, 9; P=0.1181).

Do multiple methoprene and synergized pyrethrin aerosol applications affect populations?

The overall model for the number of individuals in Petri dishes after 1, 2, 3, or 4 aerosol applications of synergized pyrethrin and Isopar M or the combination of methoprene, synergized pyrethrin, and Isopar M found significant differences (F=29.65; d.f.=7, 232; P<0.0001) (Fig. 5.3A) between treatments. Petri dishes that were treated with the combination of methoprene, synergized pyrethrin, and Isopar M had significantly fewer (F=17.44; d.f.=1, 238; P<0.0001) individuals than the Petri dishes treated with synergized pyrethrin and Isopar M. Petri dishes which received four applications of synergized pyrethrin and Isopar M or the combination of methoprene, synergized pyrethrin, and Isopar M had significantly fewer (F=63.04; d.f.=3, 236; P<0.0001) living individuals than those that received one, two, or three applications.

Treatments with less than four insecticide applications were not different from each other. The interaction between insecticide treatment and the number of aerosol applications was not significant (F=0.32; d.f.=3, 236; P=0.8093).

The overall model for the instantaneous rate of increase in Petri dishes after 1, 2, 3, or 4 aerosol applications of synergized pyrethrin and Isopar M or the combination of methoprene, synergized pyrethrin, and Isopar M found significant differences (F=19.19; d.f.=7, 232; P<0.0001) (Fig. 5.3B) between treatments. Petri dishes that were treated with the combination of methoprene, synergized pyrethrin, and Isopar M had a significantly lower (F=14.59; d.f.=1, 238; P=0.0002) instantaneous rate of increase than those that received synergized pyrethrin and Isopar m. Petri dishes that received four aerosol applications of synergized pyrethrin and Isopar M or the combination of methoprene, synergized pyrethrin, and Isopar M had a significantly lower (F=37.78; d.f.=3, 236; P<0.0001) instantaneous rate of increase than those that received one, two, or three applications, which were the same as each other. The interaction between the treatment and the number of aerosol applications received was not significant (F=1.13; d.f.=3, 238; P=0.3396).

Discussion

The instantaneous rate of increase (r_i) and the total number of living individuals, either a complete count or a subsample of the population, were used to determine if there were significant differences in populations. The first experiment explored the movement of insecticide component treated individuals into established populations. Analysis from the subsample totals and r_i showed no treatment differences but the final subsample totals and r_i showed differences in the treatments. In the second experiment

populations were hidden during the aerosol application but had access to the treated surface after the aerosol application. There were no differences between the insecticide treatments and the controls or between the sanitation treatments, clean and flour dusting. In the final experiment, treated populations with a hidden refugia and free movement between the exposure arena and the refugia, there were no significant differences between populations treated with synergized pyrethrin or the combination of methoprene and synergized pyrethrin until after the fourth aerosol application.

The two sets of analysis for the introduction of adults treated with Isopar M; methoprene and Isopar M; synergized pyrethrin and Isopar M; methoprene, synergized pyrethin, and Isopar M; or no treated adults (control) had conflicting results which makes it difficult to determine the effects of immigration of insecticide treated individuals on populations. However, the last subsample in the series prior to the final subsample had a drop in all the living individuals in the populations regardless of treatment. This indicates that the populations were declining; the r_i's were all very small, $r_i<0.001$, in most cases which supports the declining populations. Additionally, the flour in jars was heavily conditioned, the flour was yellow and contained many dead insects and cast skins (personal obs.), and was probably over utilized. It is possible that the difference in treatments seen in the final subsample was an artifact of the subsample and there are no real differences between the treatments. This suggests that the movement of Isopar M; methoprene and Isopar M, synergized pyrethrin and Isopar M; or methoprene, synergized pyrethrin, and Isopar M treated individuals into populations does not affect the population growth.

The second experiment had no differences between the populations which received the combination of methoprene, pyrethrin, and Isopar M aerosol applications or no aerosol applications (control) and there were no differences in the sanitation levels, clean or flour dusting, nested inside the treatments. The lack of difference in sanitation levels is surprising since previous work (Toews et al., 2010; chapter 4) suggest that good sanitation, small amounts of flour residue, is a critical component for reducing insect development.

Furthermore, the fact that methoprene can be readily transferred from insects to flour (chapters 2 and 3) or flour can absorb it from concrete surfaces (Arthur, 2008; Sutton et al., 2011) suggests that sanitation either by removing residues or pushing the residues into hidden areas resulting in secondary exposure to hidden populations can have an effect on population growth. The lack of differences in our study is potentially due to the extreme bimodal distribution of population numbers within each treatment group and low replication (methoprene, synergized pyrethrin, and Isopar M aerosol applications which received dust had two replicates whereas all other treatment combinations had three). Therefore, the lack of effect may be due to the lack of power in the statistics.

The final experiment showed a significantly lower number of individuals in populations that were treated with an aerosol application of the combination of methoprene, synergized pyrethrin, and Isopar M compared to those that were treated with synergized pyrethrin and Isopar M but not until after four aerosol applications. Additionally, it is not until the fourth aerosol application that the means for the insecticide treatments differ from the control populations.

The difference in the modes of action between methoprene and synergized pyrethrin may be the reason that the two treatments take some time to separate. As previously mentioned, pyrethrin is likely to kill or knockdown the exposed developmental stages (Jenson et al., 2010) but methoprene targets immature developmental stages during developmental transition periods (Beckage, 1998; Henrick, 2007) such as first-stage larva to second-stage larva or pupa to adult. If this was the only difference between the two insecticides then we would expect that the populations would stay the same indefinitely which was seen in the insecticide effects on egg development after aerosol application (chapter 4). However, pyrethrin is not stable in the environment (Barthal, 1973; Weinzierl, 1998) so its mode of action will immediately affect those individuals that are in the environment and then the insecticide will quickly degrade. Methoprene, on the other hand, is stable and persistent under indoor applications (Henrick, 2007). Therefore, over time there will be a buildup of methoprene on treated surfaces and a greater likelihood for cumulative effects on immature developmental stages in a population.

On the other hand, it is possible that the reason for the significant difference in population numbers occurring after the fourth aerosol application is due to rebound effect. Rebound occurs when insect populations return to previous numbers after a pest management strategy is employed. Dishes that received only 1 aerosol application had 8 weeks for the population levels to rebound whereas dishes which received 4 aerosol applications only had 2 weeks to recover. However, the number of dead individuals at the end of the experiment increased as the number of aerosol applications increased, e.g., 1 application of synergized pyrethrin and Isopar M (4.6±0.6), 2 (6.8±0.7), 3

(17.2±1.5), and 4 (58.3±3.6). This suggests that each subsequent aerosol application reduces a proportion of the population. Additionally, trap capture studies have shown a reduction in population fluctuations in a commercial flour mill which includes routine methoprene, synergized pyrethrin, and Isopar M aerosol applications and sanitation measures (Campbell et al., 2010ab). This finding suggest that while under our experimental design rebound could be a factor for the population differences under real-world conditions the multiple aerosol applications are a factor in suppressing populations.

In conclusion, the first two experiments imply that there is no effect from either movement of Isopar M; methoprene and Isopar M; synergized pyrethrin and Isopar M; or the combination of methoprene, synergized pyrethrin, and Isopar M treated adults into hidden populations or multiple aerosol applications of the combination of methoprene, synergized pyrethrin, and Isopar M on hidden populations. However, the confidence in these studies is low due to overutilization of resources and subsequent population declined (first experiment) and low replication resulting in low statistical power (second experiment). The third experiment, on the other hand, shows that multiple aerosol applications are more effective than one and that after four aerosol applications the combination of methoprene, synergized pyrethrin, and Isopar M is more effective on reducing population numbers than synergized pyrethrin and Isopar M. However, the role of sanitation and horizontal transfer of methoprene on *T. castaneum* populations is not clear. In the case of sanitation, further research into aerosol applications and different sanitation levels could provide further information as the role and importance of sanitation on populations. However, it may be difficult to determine

the role of horizontal transfer of methoprene on populations while considering other management tactics. Horizontal transfer of methoprene may occur at low levels but due to the high effect of the aerosol applications and the undetermined effect of sanitation it is easily overlooked or difficult to detect.

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Figures

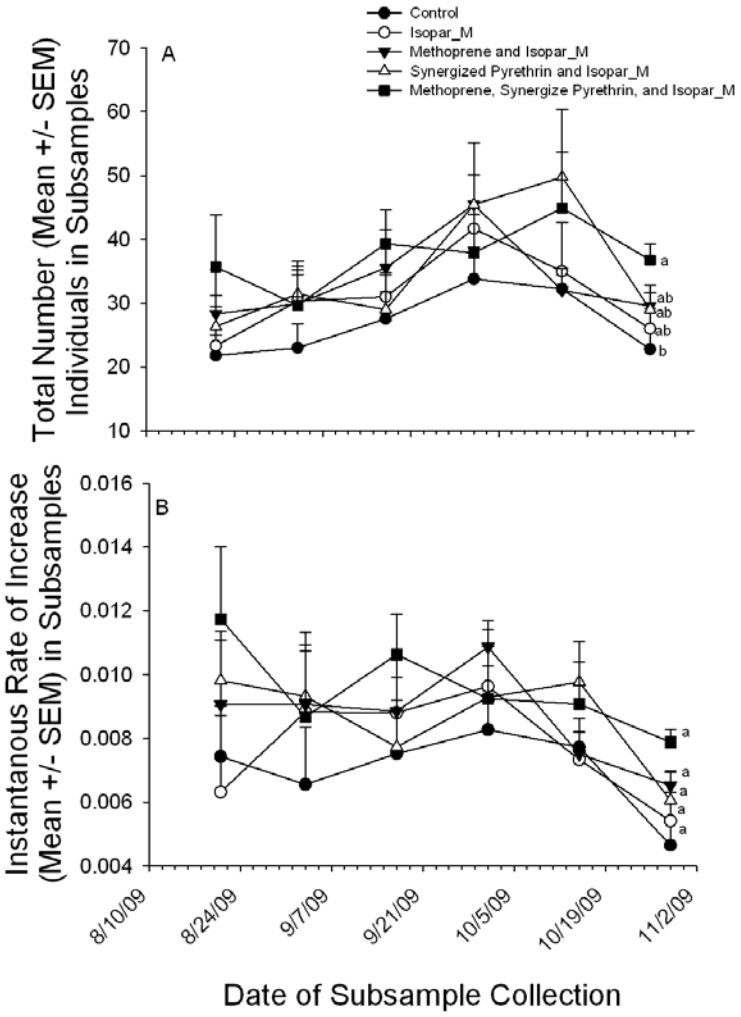

Figure 5.1 Total number (mean ± SEM) of living individuals (A) and instantaneous rate of increase (B) in the subsamples from the control populations and populations which received 10 (5 male and 5 female) dead adults treated with either Isopar M (carrier in synergized pyrethrin); methoprene and Isopar M; synergized pyrethrin and Isopar M; or the combination of methoprene, synergized pyrethrin, and Isopar M. The same lowercase letters at the end of the subsample line indicate no significant differences between the treatment means according to least square means with a Tukey-Kramer adjustment for multiple comparisons.

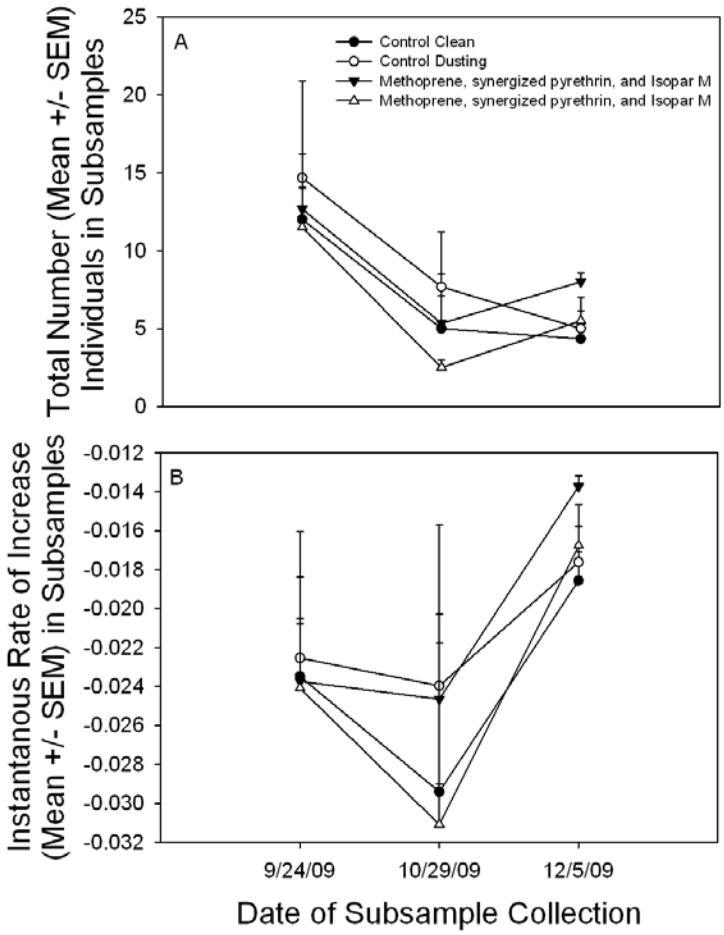

Figure 5.2 Total number (mean ± SEM) of living individuals (A) and instantaneous rate of increase (B) in the subsamples from the control populations and populations which received aerosol applications of the combination of methoprene, synergized pyrethrin, and Isopar M; each treatment had two levels of sanitation (clean received no flour dusting and dusting received a flour dusting after every aerosol application) nested within the treatment. There were no significant differences in the treatment means according to GLIMMIX.

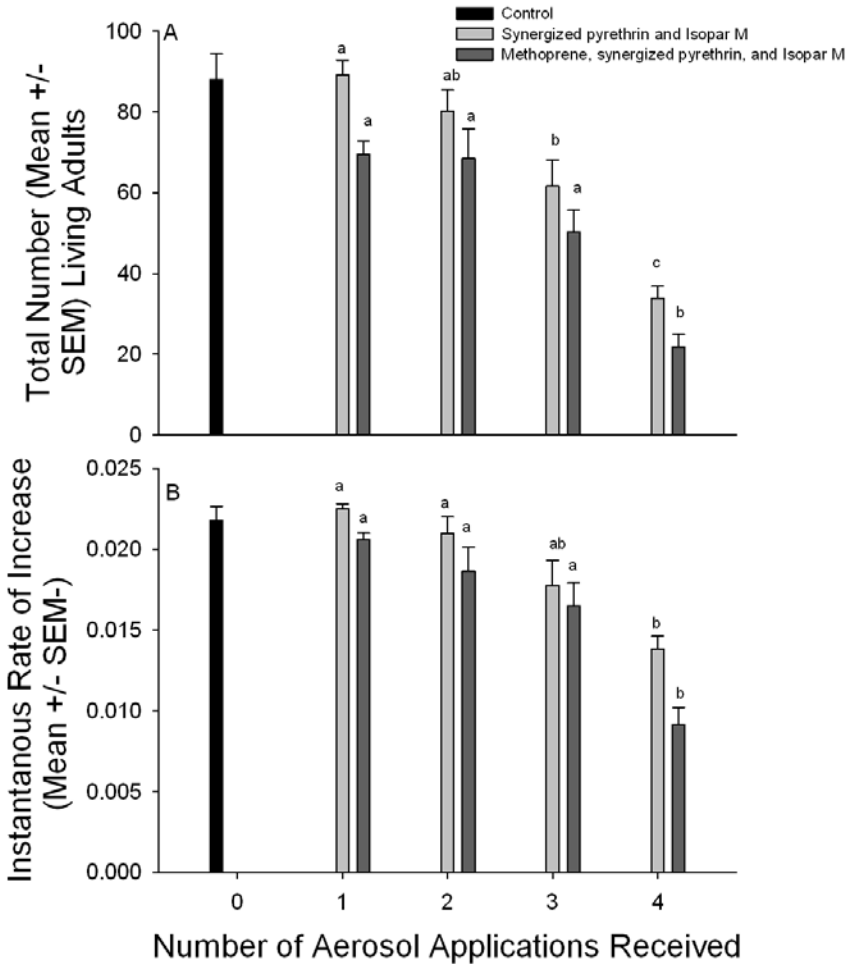

Figure 5.3 Total number (mean ±SEM) of living individuals (A) and instantaneous rate of increase (B) in *T. castaneum* populations after 1, 2, 3, or 4 aerosol application of either synergized pyrethrin and Isopar M or the combination of methoprene, synergized pyrethrin, and Isopar M. The same lowercase letters above the bars indicate no significant differences between the numbers of aerosol applications received within a treatment according to least square means with a Tukey-Kramer adjustment for multiple comparisons.

Chapter 6 - Conclusions

The phase-out of methyl bromide, a fumigant used for controlling insect pest populations in flour mill and food production facilities, has resulted in a need for alternative methods for managing pest populations. Flour mills have been increasingly relying on aerosol applications of mixed insecticides such as synergized pyrethrin and the insect growth regulator (IGR) methoprene. However, aerosol applications cannot penetrate into hidden areas where populations of *Tribolium* spp. are located. Therefore, it is not likely that using aerosol applications would be successful in reducing these populations but trapping studies from a flour mill suggest that using routine aerosol applications of synergized pyrethrin and methoprene along with sanitation suppressed resident populations of *Tribolium castaneum,* the red flour beetle. The research in this dissertation explored the potential of horizontal transfer of methoprene as a method of treating these hidden populations and the potential of synergized pyrethrin and methoprene alone and in combination to effect *T. castaneum* populations.

Horizontal transfer, the movement of insecticides from treated surfaces or individuals to untreated individuals, of methoprene (Chapter 2) can occur between treated and untreated *T. castaneum* and *T. confusum* but *T. castaneum* was more susceptible to the effects of methoprene. Pupae, early-stage larvae, and late-stage larvae that were secondarily exposed to methoprene had similar responses despite the differences in exposure times, suggesting that either the methoprene degraded over time or that there is a critical point where if methoprene is present deleterious effects such as mortality and external deformities are more likely to occur. There were several living, normal appearing adults so the potential for sub-lethal effects was explored. It

appeared that horizontal transfer of methoprene did not occur even though this experiment followed the same procedures as the other experiments and there were no differences in the number of oviposited eggs. This suggests that the horizontal transfer of methoprene may be variable.

Research described in Chapter 3 explored the mechanisms for horizontal transfer of methoprene. Cannibalization of dead, methoprene-treated developmental stages and short exposure times to methoprene-treated adults did not result in any detectable effects on late-stage larvae development to pupae and then to adults. However, exposure to multiple methoprene-treated developmental stages and flour which had been conditioned with multiple methoprene-treated developmental stages resulted in low survival and adult emergence rates and a high number of deformities. This implies a potential for methoprene to treat hidden populations by the transfer of flour across contaminated surfaces and into cracks, crevices and other hidden areas where *T. castaneum* populations are located.

The potential for the components of aerosol applications of methoprene and synergized pyrethrins, individually and in combination, to affect *T. castaneum* individuals (chapter 4) was explored. There were no effects (mortality or decrease in progeny) from the use of the carrier used in commercial formulations of synergized pyrethrins. All *T. castaneum* adults that were directly exposed to synergized pyrethrins, either alone or in combination with methoprene, were knocked down after the aerosol application, but most of them recovered with no subsequent reduction in progeny production. Exposure of eggs to pyrethrins alone resulted in reduced egg hatch, but the addition of methoprene did not result in any further reductions in egg hatch. Three

depths of flour were used to determine the effect of sanitation on the insecticide efficacy on immature development. When a low amount of flour was present, development was reduced, as the flour depth increased the combination of methoprene and synergized pyrethrin reduced development to the adult stage. Although this result suggests that both sanitation and insecticides can be used to reduce the pest populations, it is possible that at some depth of flour the insects could potentially escape the effect of insecticides.

Research described in Chapter 5 explored the ability of these insecticides to effect hidden populations. The direct introduction of adults treated with synergized pyrethrin into established colonies reduced populations, suggesting that the presence of adult beetles exposed to synergized pyrethrin can affect hidden populations through transfer of toxicant to the resident populations. However, when beetle populations were provided with hidden refugia, sanitation measures directed at the site where beetles were exposed to aerosol applications did not have any detectable effects on the resident population. However, multiple aerosol applications on a population with hidden refugia where the insects could move between the exposure arena and hidden refugia during aerosol applications did have an effect on the population. Populations that received four applications of methoprene and synergized pyrethrin were significantly lower than those which received synergized pyrethrin alone. This result implies that the addition of methoprene to synergized pyrethrin increases the suppression of populations. However, the role of horizontal transfer of methoprene and sanitation on *T. castaneum* populations is still not clear.

In conclusion, the research in this dissertation has increased the knowledge of aerosol applications of methoprene and synergized pyrethrin and the potential of sanitation measures on *T. castaneum* development. This knowledge can aid insect pest managers in making better decisions in controlling *T. castaneum* and other stored product pest populations.

Appendix A - Chapter 2: *T. confusum* Adultoid Deformity

Figure 6.1 Ventral view of a *T. confusum* that as a late-stage larva was exposed to a methoprene treated developmental stage (late-stage larva, pupa, or adult) and flour and exhibited external deformities in the transition between pupa and adult.

Figure 6.2 Lateral view of the same individual presented above.

Appendix B - Chapter 2: *T. confusum* Adult Deformity

Figure 6.3 Lateral view of a *T. confusum* that as a late-stage larva was exposed to a methoprene treated developmental stage (late-stage larva, pupa, or adult) and flour and exhibited severe external deformities after adult emergence.

Appendix C - Chapter 3: *T. castaneum* Pupa Deformity

Figure 6.4 Ventral view of a *T. castaneum* that as a late-stage larva was exposed flour that was treated with 5 methoprene developmental stages (late-stage larvae, pupae, or adults) and exhibited external deformities in the pupal stage.

Appendix D - Chapter 3: *T. castaneum* Unknown Developmental Stage Deformity

Figure 6.5 Lateral view of a *T. castaneum* that as a late-stage larva was exposed to flour that was conditioned with 5, 15, or 30 methoprene treated developmental stages (late-stage larvae, pupae, or adults) and during development exhibited severe external deformities.

Appendix E - Chapter 5: Diagram for Utility Shed

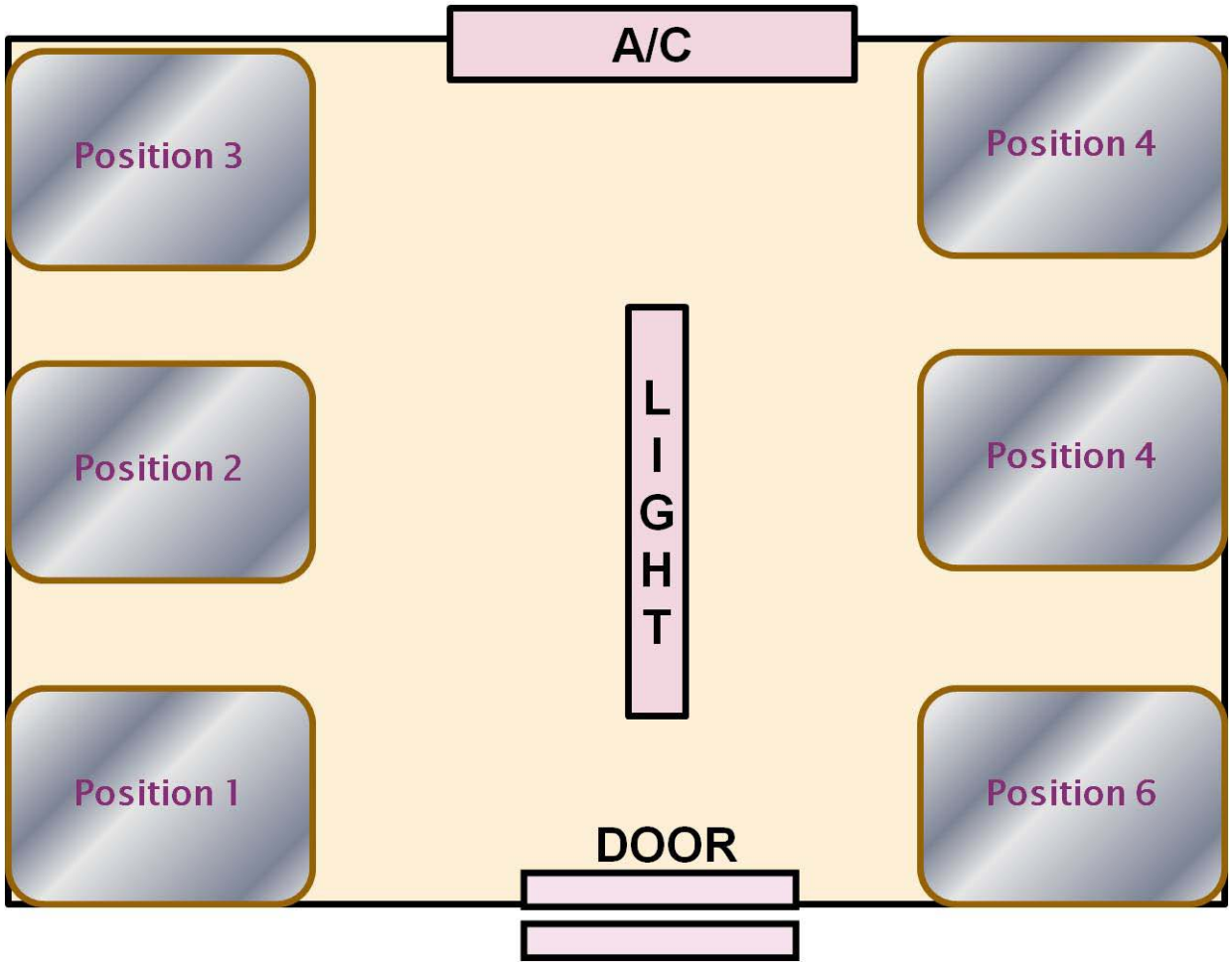

Figure 6.6 The layout, drawing not to scale, for the fixed structures and the experimental units in the utility shed used in experiment three; the numbers identify the general locations where Petri dishes were placed inside the shed during the aerosol applications.