For Submission:

Journal of Stored Products Research

Submitting Author

Special Issue

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Effects of Methoprene and Synergized Pyrethrin Aerosol Applications on *Tribolium*
castaneum (Herbst) Populations

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24 Abstract

25 The occurrence of horizontal transfer of the insect growth regulator (IGR) methoprene on
26 confined populations of *Tribolium castaneum* (Herbst), either with or without hidden refugia,
27 was determined through a series of experiments. Multiple applications were made with the IGR
28 alone or combined with synergized pyrethrin, and compared to untreated controls which received
29 no insecticide applications or were treated with the carrier Isopar M that was a component of the
30 pyrethrin formulation. The total number of living beetles from test colonies inoculated with
31 adults that were treated with Isopar M or with no adults (control) was significantly greater than
32 those colonies that were inoculated with adults treated with either synergized pyrethrin or
33 methoprene ($P < 0.05$). There was no difference in the number of living individual in the larval,
34 pupal, and adult stages and the instantaneous rate of increase (r_i) in established populations
35 treated with methoprene and containing a hidden refugia compared to those which received the
36 pyrethrin applications ($P \geq 0.05$). Sanitation levels of two different flour quantities nested inside
37 the treatment groups also had no effects ($P \geq 0.05$), suggesting that populations in hidden refugia
38 can persist even with multiple applications of methoprene and synergized pyrethrin. Populations
39 with an accessible hidden refugia that were exposed to synergized pyrethrin and methoprene had
40 a lower number of living adult and a lower r_i value than populations that were exposed to
41 synergized pyrethrin alone ($P < 0.001$). Additionally, populations which received one, two, or
42 three aerosol applications had similar numbers of living adults and r_i but were significantly
43 different from populations which received four aerosol applications. Results suggest multiple
44 applications of methoprene and synergized pyrethrin could be more effective than synergized
45 pyrethrin alone for control of *T. castaneum*.

46 **Keywords:** *Tribolium castaneum*, methoprene, synergized pyrethrin, population impact, aerosol
47 application

1. Introduction

Tribolium castaneum (Herbst), the red flour beetle, is a cosmopolitan pest of 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 potential costs involved with control measures. *Tribolium castaneum* populations can be difficult to control since they exploit cracks and crevices and refugia inside equipment or in the building structure (Campbell et al., 2004; Toews et al., 2005). One component of *T. castaneum* management is through the use of aerosol insecticides (Peckman and Arthur, 2006). Aerosols can disperse inside a structure but may not penetrate into hidden areas where refugial populations could persist (Toews et al., 2009; 2010). However, regular aerosol applications of methoprene and synergized pyrethrin as part of an integrated pest management (IPM) program will give some suppression of resident populations (Campbell et al., 2010ab; Toews et al. 2010).

The combination of methoprene and synergized pyrethrin can be an effective aerosol application because methoprene, which is an insect growth regulator, targets immature insects through disruption of normal development (Beckage, 1998; Henrick, 2007) and synergized pyrethrin results in knockdown and direct kill of all exposed life stages (Jenson et al., 2010). Methoprene will persist on exposed flooring surfaces and the exterior of different materials used to package flour bags for shipment, which results in long-term efficacy and potential gradual control of resident populations (Sutton et al., 2011). The immediate effects of synergized pyrethrin and methoprene aerosol applications on different developmental stages of *T. castaneum* have been evaluated (Arthur, 2008; Toews et al., 2010; Tucker et al., 2014abc). While the results from individual toxicological studies provide an indication of the insecticide toxicity, this

information may not accurately reflect the efficacy of the insecticide on the suppression of the insect populations (Stark and Banks, 2003; Toews et al., 2005).

Insecticides applied to exposed surfaces may migrate into hidden areas through a variety of mechanisms. Horizontal transfer of methoprene from a treated individual to another individual can occur and that contact and contamination of a food-source (flour) is a potential mechanism (Tucker et al., 2014abc). Although synergized pyrethrin can have an initial high knockdown effect on adult *T. castaneum* (Tucker et al., 2014c), and also the related *T. confusum* Jacquelin DuVal, adults that are knocked down by pyrethrins can recover (Kharel et al., 2014ab). In addition, recovery can be more extensive in the presence of a food source (Arthur and Campbell, 2008; Kharel et al., 2014ab).

Results of the studies described above also suggest there is limited effect from a single application of an aerosol, but flour mills and food facilities often 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 et al., 2010ab). Therefore, the objectives of this test were to determine: 1) if immigration of adults treated with pyrethrin alone or with methoprene affected overall population levels, 2) effects of multiple aerosol applications when untreated refugia were present, and 3) effects of those multiple applications when beetles could move freely into and out of the refugia.

2. Materials and Methods

2.1. Effect of immigration of adults treated with methoprene and synergized pyrethrin

A field strain established from collections made from a flour mill in 2004 was used in the experiments (Romero et al., 2010). Cultures of this field strain have been maintained at the USDA-ARS Center for Grain and Animal Health Research (CGAHR) since that time. These

cultures were reared inside an incubator set at $28.3 \pm 0.0^\circ\text{C}$, $46.3 \pm 0.1\%$ RH and 14/10 (day/night) photoperiod, and rearing procedures have been previously described in detail (Tucker et al., 2014abc). Voucher specimens of adults used in this study were deposited in the Kansas State University Museum of Entomological and Prairie Arthropod Research, Manhattan, KS, USA, under voucher number 226.

Experimental populations of *T. castaneum* were created by placing 5 male and 5 female adults from the cultures described above, along with 200 g of flour, in each of fifteen 0.95L glass jars, and holding these jars for 3 months to allow a resident population to develop. Adult *T. castaneum* were collected by sexing groups of 1-2-week-old adults of each sex from the insect cultures described in the previous sentence. Sexing was done by determining if the hind femur contained a setiferous patch; males have this patch and females do not (Hinton, 1942).

Exposure arenas were created in the bottom portion of plastic Petri dishes (approximate area of 62 cm^2), by mixing a dry concrete material (Rockkrite®, Hartline Products, Co., Inc., Cleveland, OH, USA), with tap water. This mixture was poured into each Petri dish bottom to a depth of about 0.125 cm. Then, approximately 50 males and 50 females were placed in each of two separate 90 x 15 mm (62 cm^2) Petri dishes with a concrete bottom (Rockkrite®, Hartline Products, Co., Inc., Cleveland, OH, USA). The Petri dishes were then placed in one of four plastic rectangular boxes with a layer of concrete on the bottom (length $56.5 \pm 0.2\text{ cm}$, width $34.6 \pm 0.3\text{ cm}$, and height after addition of concrete $15.5 \pm 0.1\text{ cm}$) for the simulated aerosol applications. Separate boxes were used for four different insecticide treatments, which were the carrier Isopar M (Entech Systems, Kenner, LA, USA); methoprene (Diacon® II, 33.6% active ingredient (AI), Central Life Sciences, West Schaumburg, IL, USA) and Isopar M; synergized

pyrethrin (Entech Fog-10, 1.0% AI, Entech Systems, Kenner, LA, USA) and Isopar M; and the combination of methoprene, synergized pyrethrin, and Isopar M..

The amount of the insecticide 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 were previously reported by Tucker et al. (2014c), but this translates into 0.0045 mg AI of methoprene in Isopar M and 0.02 mg AI of synergized pyrethrin g/cm² in Isopar M; the same amount AI was present in the combination of methoprene, synergized pyrethrin, and Isopar M. Insecticide was applied to the concrete surface of the box, including the dishes containing the insects, using an artist's air-brush (E Badger Air Brush 100 LGM, Badger Air-Brush Company, Franklin Park, IL, USA) to spray the insecticide onto the surface. The boxes were allowed to air-dry for one hour, and then the process was repeated twice. The insects were placed in new Petri dishes and frozen, for 24 h at 54.0±0.2% RH and -16.8±0.0 °C. The adults were frozen to reduce degradation of insecticides (Henrick, 2007) and to standardize treatment conditions since synergized pyrethrin will cause adult knockdown or mortality whereas methoprene does not. Each treatment process was replicated three times with three separate spray treatments and these treatments were repeated every two weeks for six weeks.

The effect of immigration of these treated adults on the established populations was evaluated by adding treated beetles every two weeks to the culture jars containing *T. castaneum* that were established as previously described. Five males and five females from each treatment group were placed in each respective designated experimental jar with established populations 9 jars for each of the 4 insecticide treatments plus the untreated control, 45 jars total). An

individual jar was gently agitated for 1 min to simulate movement of the treated beetles 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 previous experiments showed that the water controls had little or no effect on the insect development (Tucker et al., 2014abc).

Samples were collected from jars immediately prior to adding the treated insects and then at two week intervals for four months. Jars were sampled by mixing the jars to evenly distribute the developmental stages, and using a plastic spoon to remove 1 g of flour from the center of the jar. The number of beetles in the larval, pupal, and adult stages, along with the number of dead adults, was tabulated and recorded. Samples were not placed back into the colony jars. At the end of the experiment a larger 2 g subsample of the flour was taken in the same manner and the numbers of live and dead beetles tabulated as previously described.

The data (total number of living beetles) from the 1-g samples were 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 the Tukey-Kramer means separation procedure 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 of the Statistical Analysis System (SAS v 9.2 software, SAS Institute, Cary, NC, USA) for both the total number of beetles and r_i . Significant differences among treatments ($P < 0.05$) were determined using the Tukey means separation test as an option under the GLM Procedure.

2.2. Effects of multiple methoprene and synergized pyrethrin aerosol applications and sanitation

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 Petri dish with a surface area of 62 cm² was inserted into the concrete before it dried. 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 concrete surface by 2 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 pieces of filter paper (Whatman Grade 1, GE Healthcare Co., Little Chalfont, BKM, UK) strips (2 x 2 cm) were placed between the flour and 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 from the base colony to each plastic container with flour. The insecticide treatments were initiated one month after establishing the population.

The amount of insecticide applied to each arena was determined as described for Experiment 1. Prior to insecticide applications, the number of beetles outside the refugia was counted and all but 5 adults were returned to the Petri dish refugia. The refugia was 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 5 beetles left out of the refugia were used to simulate the small percentage of a population that might be directly exposed during aerosol treatment. There was a 1-hr drying 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 the refugia were covered with the 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 2 wks for a total of 8 applications. Nested within each treatment and control treatments were 2 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 immediately 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 cause the beetles to spend more time out of the refugia and potentially result in increased exposure to treated surfaces. Also, 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 (10, 15, and 20 wks) during the experiment. 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 live beetles in the larval, pupal, and adult stages, and the number of dead beetles, was tabulated and recorded. Subsamples were not returned to the Petri dish after counting. At the end of the experiment, after the last subsample was taken all of live beetles of the life stages described above, and the number of dead adults, was tabulated and recorded. The r_i was calculated for each subsample and the final population counts and those counts and r_i values were analyzed as described above. The number of living beetles and r_i in insecticide treatments were compared with those of the controls, and the same comparisons were made for the two different sanitation levels.

2.3. Effect of multiple methoprene and synergized pyrethrin aerosol applications

This experiment had the same objective as the previous experiment but was slightly different in that a smaller arena was used in order to increase the replication number, and the refugia were left open during aerosol applications. Leaving the refugia open allowed potential for mobile life stages of *T. castaneum* to escape the effects of direct aerosol exposure. The expanded experimental design also allowed for potential dispersal of the insecticides into the hidden area and thereby secondarily exposing previously unexposed beetles. Finally, the experimental arenas were placed inside a small shed, which required the use of a hand-held applicator to apply the aerosol. Procedures are described below.

The population arenas were made with a plastic Petri dish (150 x 25 mm; 176 cm² area of the bottom) containing a layer of concrete in the bottom, prepared as described above. The

inner sides of the Petri dishes were coated with liquid Teflon (Fluon AD-1, Northern Products, Inc., Woonsocket, RI, USA) to confine the beetles to the arenas. Refugia for each arena were created by placing 3.5 g of flour in a 7.4 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. To facilitate climbing by the beetles the front of the plastic cap was sanded with a piece of 100 grit aluminum oxide resin/gum sandpaper (3M, Construction and Home Improvement Markets Division, St. Paul, MN, USA) and the interior side of the plastic cap was sanded with a 3/8 inch (0.95 cm) Dremel Multipro (Model 395T6, Racine, WI, USA). Preliminary studies showed that beetles could enter and exit the vial after the above modifications to the vial cap were performed.

Tribolium castaneum populations were established by placing two virgin males and females (founders) inside the glass vial in the Petri dish. Insecticide applications started 2 weeks after the founders were added to glass vials. Dishes were held in an incubator at 31°C and 60% 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 6 Petri dishes were placed in each shed, 3 on the left side and 3 on the right side of the shed. The lids were removed from the Petri dish and placed face up next to the dish. The labeled rate of synergized pyrethrin (approximately 30 ml / 28.3 m³) in Isopar M, or combination of methoprene (3 ml / 283.2 m³) added to the labeled rate of synergized pyrethrin in Isopar M, was used to treat the shed. This resulted in a calculated application of 450 mg AI of synergized pyrethrin in Isopar M applied to each shed and 389 mg AI of methoprene was added to the synergized pyrethrin and

Isopar M to sheds that received the combination of methoprene, synergized pyrethrin, and Isopar M. Assuming a 75% deposition to the surface of the floor, each dish that was in a shed treated with synergized pyrethrin in Isopar M would theoretically receive 3.5 mg AI and dishes that received the combination would also receive 3.1 mg AI of methoprene. A hand-held aerosol applicator (FogMaster Jr, Model 533010CA, The Fogmaster Corp., Deerfield Beach, FL, USA) was used to apply the insecticides in the sheds.

The aerosol insecticides were applied by a person holding the applicator, walking down the middle of the shed between the 2 rows of Petri dishes, and moving the aerosol applicator from left to right as the aerosol was dispensed. 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 1-hr drying period the dishes were removed and placed in the incubator. Treatment dishes received 1, 2, 3, or 4 aerosol applications at 2 wk intervals. The control Petri dishes were not sprayed and were held in the incubators. There were 4 replicate aerosol applications for each treatment. The controls and insecticide treatments which received 1, 2, or 3 insecticide applications had 48 dishes total, but the insecticide treatments which received four aerosol applications had 96 dishes (dishes originally slated for more aerosol applications were combined into the 4 application group due to the status of the insect populations).

Prior to the aerosol application, the number of dead and living adults outside of the glass vial were counted and recorded for all Petri dishes. 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 (eggs, larvae, and pupae). A repeated measures test, procedure GLIMMIX (SAS software) was used to analyze the number of living adults observed in the open areas of the exposure arena prior to the aerosol applications. The r_i was calculated, using total living adult counts, and analyzed with GLM as described above for each Petri dish. The GLM model included the number of aerosol applications and the insecticide treatments to determine their effect on populations. The data from the controls were not analyzed as they 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. The total number of dead adults at the end of the experiment was analyzed using the GLM Procedure of SAS, as described for the previous two experiments.

3. Results

3.1. Effect of immigration of adults treated with methoprene and synergized pyrethrin

There were significant differences in the total number of live beetles in subsamples from the treatments, control; Isopar M; methoprene and Isopar M; and synergized pyrethrin, methoprene, and Isopar M, (GLIMMIX: $F=2.9$; $d.f.=4, 240$; $P=0.022$) (Fig. 1A). The number of live beetles in 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 ($F=5.0$; $d.f.=5, 240$; $P<0.001$) was significant but the interaction between treatment and sample date was not ($F=0.50$; $d.f.=20, 240$; $P=0.964$). The decline in the number of total beetles at the last sample date suggests that the jars were becoming over utilized and the population had reached carrying capacity. This overutilization of the jars could explain why the control jars had the lowest populations as they would have no mortality related to insecticide treated beetles, therefore, their populations would have reached capacity and declined

faster than the other treatments. At the end of the experiment, there were significant differences between treatments in the mean number of living beetles ($F=4.1$; $d.f.=4, 37$; $P=0.007$) in the 2 g subsamples (Fig. 1A). The total numbers in the controls versus the colonies and in the colonies receiving the beetles treated with Isopar M (491 ± 44 and 488 ± 26) were greater than the total numbers in the synergized pyrethrin plus Isopar M colonies (368 ± 26). However, there were no differences between the insecticide treatments themselves.

The instantaneous rate of increase (r_i) was not significantly different between the treatments ($F=2.1$; $d.f.=4, 240$; $P=0.080$) (Fig. 1B). In the overall model sample date ($F=2.60$; $d.f.=5, 240$; $P=0.026$) was significant but the interaction between insecticide treatment and sample date was not ($F=0.5$; $d.f.=20, 240$; $P=0.978$). The mean r_i , calculated for the final 2 g subsample, was significantly different between the treatments. The r_i in the colonies that had received the adults treated with the Isopar M carrier (0.022) or had received the untreated adults (0.022) was greater ($F=4.1$; $d.f.=4, 37$; $P=0.007$) than the r_i in the synergized pyrethrin and Isopar M (0.02) colonies. There was no difference in r_i between the insecticide treatments. The insecticide treatments did reduce the intrinsic growth rate compared to the controls but overall numbers of live beetles in the treatments were still high.

2.2 Effects of multiple methoprene and synergized pyrethrin aerosol applications and sanitation

The total number of living beetles in the subsamples (Fig. 2A) was not significantly different with respect to treatment ($F=0.1$; $d.f.=1, 21$; $P=0.770$), sanitation ($F=0.01$; $d.f.=1, 21$; $P=0.963$), or the sample date ($F=1.4$; $d.f.=1, 21$; $P=0.255$). Additionally, there were no significant interactions ($P\geq 0.05$) between the treatment, sanitation, and sample date. The calculated r_i (Fig. 2B) was not significantly different for the treatment ($F=0.01$; $d.f.=1, 21$; $P=0.931$) or the level of sanitation ($F=0.02$; $d.f.=1, 21$; $P=0.887$), but was significantly different

for sample date ($F=4.6$; $d.f.=2, 21$; $P=0.023$). None of the interactions between the treatment, sanitation, and sample date were significant ($P \geq 0.05$). Hence, none of the insecticide treatments appeared to have any effect on the *T. castaneum* populations.

There were no significant differences ($F=1.1$; $d.f.=3, 7$; $P=0.407$) for the total count of living beetles after either no insecticide applications (control 235 ± 103) or multiple exposures to the combination of methoprene, synergized pyrethrin, and Isopar M (148 ± 53) and nested within each insecticide treatment a clean concrete arena (155 ± 40) or arena with a dusting of flour (244 ± 127) to represent different levels of sanitation. For the specific factors in the model there were no significant differences for the treatment ($F=0.7$; $d.f.=1, 9$; $P=0.431$), sanitation level ($F=0.4$; $d.f.=1, 9$; $P=0.621$), or interaction between the treatment and sanitation level ($F=2.3$; $d.f.=1, 9$; $P=0.173$). The r_i also did not show significant differences in the overall model ($F=3.4$; $d.f.=3, 7$; $P=0.082$) between the insecticide and sanitation levels. The clean sanitation level (0.008) was not different ($F=3.1$; $d.f.=1, 9$; $P=0.120$) from the sanitation level which received a flour dusting (0.008). The interaction between the treatment and the sanitation level was not significant ($F=3.2$; $d.f.=1, 9$; $P=0.118$).

3.3. Effect of multiple methoprene and synergized pyrethrin aerosol applications

The number of living adults in the open area of the exposure arenas was not significantly different between treatments and the number of aerosol applications prior to the first and second aerosol applications ($F=1.1$; $d.f.=7, 232$; $P=0.358$, $F=1.8$; $d.f.=7, 232$; $P=0.093$; respectively). There were no significant differences for the interaction for the number of aerosols, treatment, or between the number of aerosols and the treatment prior to the first aerosol application (aerosol: $F=1.1$; $d.f.=3, 236$; $P=0.367$; treatment: $F=2.7$; $d.f.=1, 238$; $P=0.099$; aerosol by treatment: $F=0.6$; $d.f.=3, 236$; $P=0.608$). However, this was expected since all of the treatments would be

the same prior to receiving the first aerosol application. Conversely, the interaction between the number of aerosols and the treatment was significantly different prior to the second aerosol application but this is likely an artifact of the data since all of the treatments would have received one aerosol application (aerosol: $F=0.5$; $d.f.= 3, 236$; $P= 0.688$; treatment: $F= 0.6$; $d.f.= 1, 238$; $P= 0.455$; aerosol by treatment: $F=3.4$; $d.f.= 3, 236$; $P= 0.017$). However, the numbers of adults between treatments and the number of aerosol applications prior to the third and fourth aerosol application and two weeks after the fourth aerosol application was different (3rd $F= 2.3$; $d.f.= 7, 232$; $P= 0.032$, 4th $F= 30.2$; $d.f.= 7, 232$; $P\leq 0.001$, two weeks after 4th $F= 61.4$; $d.f.= 7, 232$; $P\leq 0.001$). In the last three observation points there were fewer beetles in the open area of the exposure area in the dishes treated with the combination of methoprene, synergized pyrethrin, and Isopar M than in the synergized pyrethrin and Isopar M (3rd $F= 5.7$; $d.f.= 1, 238$; $P= 0.018$, 4th $F= 4.6$; $d.f.= 1, 238$; $P= 0.032$, two weeks after 4th $F= 7.0$; $d.f.= 1, 238$; $P= 0.008$). Additionally, there were significant differences between the number of aerosol applications received in the sampling periods prior to the fourth aerosol application and two weeks after the fourth aerosol application but no differences prior to the third aerosol application (3rd $F= 2.0$; $d.f.= 3, 236$; $P= 0.117$, 4th $F= 67.1$; $d.f.= 3, 236$; $P\leq 0.001$, two weeks after 4th $F= 139.5$; $d.f.= 3, 236$; $P\leq 0.001$). There were no significant differences in the number of adults in the open area for the interaction between the treatment and the number of aerosol applications received prior to the third and fourth aerosol application and two weeks after the fourth aerosol application (3rd $F= 1.3$; $d.f.= 3, 236$; $P= 0.263$, 4th $F= 1.85$; $d.f.= 3, 236$; $P= 0.143$, two weeks after 4th $F= 1.3$; $d.f.= 3, 236$; $P= 0.260$).

The overall model for the number of beetles in Petri dishes after one, two, three, or four aerosol applications of synergized pyrethrin and Isopar M or the combination of methoprene,

synergized pyrethrin, and Isopar M was significant between treatments ($F=29.6$; $d.f.=7$, 232; $P<0.000$) (Fig. 3A). Petri dishes that were treated with the combination of methoprene, synergized pyrethrin, and Isopar M had significantly fewer beetles than the Petri dishes treated with synergized pyrethrin plus Isopar M ($F=17.4$; $d.f.=1$, 238; $P<0.001$). Petri dishes which received four applications of synergized pyrethrin and Isopar M or the combination of methoprene, synergized pyrethrin, and Isopar M had fewer living beetles than those that received one, two, or three applications ($F=63.0$; $d.f.=3$, 236; $P<0.001$). 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 ($F=0.3$; $d.f.=3$, 236; $P=0.809$).

The overall model for the r_i showed significant differences between treatments ($F=19.2$; $d.f.=7$, 232; $P<0.001$) (Fig. 3B). Petri dishes that were treated with the combination of methoprene, synergized pyrethrin, and Isopar M had a lower r_i than those that received synergized pyrethrin and Isopar M ($F=14.6$; $d.f.=1$, 238; $P=0.002$). 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 lower r_i than those that received one, two, or three applications ($F=37.8$; $d.f.=3$, 236; $P<0.001$). The interaction between the treatment and the number of aerosol applications received was not significant ($F=1.1$; $d.f.=3$, 238; $P=0.340$).

The number of dead adults was significantly different between treatment times ($F=76.2$; $d.f.=7$, 232; $P<0.001$) (Fig. 4). Petri dishes which received four aerosol applications had more dead adults than the dishes which received fewer aerosol applications ($F=177.6$; $d.f.=3$, 236; $P<0.001$). There was no difference in the number of dead adults between insecticides ($F=0.4$;

$d.f.=1, 238; P=0.534$) and there was no interaction between the number of aerosol sprays and the insecticide applied ($F=0.04; d.f.=3, 236; P=0.988$).

4. Discussion

The first experiment of this study explored the movement of insecticide component treated beetles 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. This last subsample had a decline of living beetles in the populations regardless of treatment, which was reflected in the small r_i values. Additionally, the flour in the jars was yellow and contained many dead insects and cast skins, which indicated the flour was over utilized by the *T. castaneum* populations. Hence, the introduction of treated individuals had little effect because the populations had already exploited the available food resource.

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. The lack of difference in sanitation levels is surprising since the presence of a food source reduces efficacy of pyrethrin aerosol (Kharel, 2014b; Toews et al., 2010). Furthermore, methoprene can be readily transferred from insects to flour (Tucker et al. 2014ab), and can be absorbed by flour placed on a concrete surface treated with methoprene (Arthur, 2008; Sutton et al., 2011). Removing residues or pushing the residues into hidden areas and thereby resulting in secondary exposure to hidden populations was expected to have an effect on population growth in experiment 2. The lack of differences in our study could relate to either to the extreme bimodal distribution of population numbers within each treatment group or the uneven distribution of the populations within the treatment arenas.

The final experiment gave an indication that repeated applications of an aerosol could result in lower *T. castaneum* populations, but this effect was again reduced by the presence of what could be considered high population levels. In our test, the effects were not significant until the fourth application. The difference in the modes of action between methoprene and synergized pyrethrin may be one reason why it took several weeks until a treatment difference occurred. As previously mentioned, pyrethrin will give immediate knockdown or kill of exposed adults and exposed immature stages of stored product insects (Jenson et al., 2010) but methoprene targets immature developmental stages during developmental transition periods (Beckage, 1998; Henrick, 2007). However, pyrethrin is not stable in the environment (Barthel, 1973; Weinzieryl, 1998) and quickly degrades, while methoprene is stable and persistent when applied indoors (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.

In conclusion, the occurrence of horizontal transfer of methoprene into resident *T. castaneum* populations was not clear in our laboratory study and confounded by multiple factors, including the overall population levels. Earlier studies have shown that populations may continue to persist even though there appears to be an obvious effect from repeated aerosol applications (Toews et al., 2010). The presence of food either during or after an aerosol application appears to result in increased survival of *T. castaneum* and also the related *Tribolium confusum* Jacqueline Do Val, the confused flour beetle (Arthur, 2008; Arthur and Campbell, 2008), yet the effects of sanitation are difficult to quantify in simulated or actual field studies.

433 **Acknowledgments**

434 We thank Douglas Van Gundy of Central Life Sciences for supplying methoprene and Robert
435 Drudge of Entech Systems for supplying the Isopar M and Entech Fog-10. We thank Casey
436 Persson, Rich Hammel, Brian Barnett, Dylan Robinson, Konner Cool, and Ken Friessen for
437 technical support; and Dr. Leigh Murray for statistical advice. We also thank Dr. Bhadriraju
438 Subramanyam, Dr. Susan Brown, and Dr. David Margolies for comments on earlier drafts.
439 Research was partially funded by the U.S. Department of Agriculture, National Institute of Food
440 and Agriculture (NIFA) Methyl Bromide Transitions program (grant number 2010-51102-
441 21660). This is KAES contribution No. 14-369-J. Mention of trade names or commercial
442 products in this publication is solely for the purpose of providing specific information and does
443 not imply recommendation or endorsement by the U. S. Department of Agriculture or Kansas
444 State University. USDA and Kansas State University are equal opportunity providers and
445 employers.

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531 **Figure Captions**

532 Figure 1. The total (mean $\pm$ SEM) of living beetles (A) and instantaneous rate of increase (r_i) (B)
 533 in the subsamples populations which received 10 (5 male and 5 female) dead adults treated with
 534 either Isopar M (open circle), methoprene and Isopar M (filled inverted triangle), synergized
 535 pyrethrin and Isopar M (open upright triangle); the combination of methoprene, synergized
 536 pyrethrin, and Isopar M (filled square), or dead untreated controls (filled circle). Significant
 537 differences ($P < 0.05$) on the last sample date denoted by different lower case letters (least square
 538 means with a Tukey-Kramer adjustment for multiple comparisons).

539
 540 Figure 2. The total number (mean $\pm$ SEM) of the of living beetles (A) and instantaneous rate of
 541 increase (r_i) (B) in the subsamples from the control populations (clean, filled circle; flour
 542 dusting, open circle) and populations which received aerosol applications of the combination of
 543 methoprene, synergized pyrethrin, and Isopar M (clean, filled triangle; flour dusting, open
 544 triangle). No significant differences between cleaning or the two sanitation levels.

545
 546 Figure 3. The total number (mean $\pm$ SEM) of living beetles (A) and instantaneous rate of increase
 547 (r_i) (B) in *T. castaneum* populations after 1, 2, 3, or 4 aerosol applications of either synergized
 548 pyrethrin and Isopar M (light bar) or the combination of methoprene, synergized pyrethrin, and
 549 Isopar M (grey bar). Starting control populations noted with black bar. Significant differences
 550 between the two insecticide treatments denoted by different lower case letters, significant
 551 differences between times for each insecticide noted by different upper case letters ($P < 0.05$,
 552 Tukey-Kramer adjustment for multiple comparisons).

553

554 Figure 4. The total number (mean $\pm$ SEM) of dead beetles in *T. castaneum* populations after 1, 2,
555 3, or 4 aerosol applications of either synergized pyrethrin and Isopar M (light bar) or the
556 combination of methoprene, synergized pyrethrin, and Isopar M. (grey bar). Number of dead
557 beetles in starting control populations noted with black bar. Significant differences between the
558 times noted by different lower case letters ($P < 0.05$, Tukey-Kramer adjustment for multiple
559 comparisons), no difference between treatments.

Figure 1

Figure 2

Figure 3

Figure 4